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PRINCIPAL INVESTIGATOR: Roel Nusse, Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305

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Roel Nusse, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
Stanford, California 94305

e-mail:
rnusse@cmgm.stanford.edu

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The work done in this grant was aimed at elucidating the function of the *Wnt* signaling molecules in cancer, in particular at isolating a receptor. *Wnt* proteins are secreted and play important growth controlling roles, in particular in the mammary gland. They can act as oncogenes in mouse mammary tumors. Using cell biological and biochemical approaches we succeeded in finding a receptor for *wingless* called *frizzled*. We have further characterized this receptor, and we have obtained genetic proof that this receptor is required for wingless signaling in vivo by generating mutations in the receptor gene.

We have also isolated a novel gene in *Drosophila* that modifies *wingless* signaling, tartaruga This gene is highly conserved, encodes a component of histone de-acetylase activity and may be involved in Wnt regulation in vertebrates as well.

We expect that these findings are not only going to be important for basic research but also for the treatment of cancer, including breast cancer. Specific cell surface receptors, such as the Frizzleds, are important targets for therapeutic intervention. Moreover, point mutations in those receptors may occur in human cells and lead to abnormal growth.

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(5) Introduction

Wnt genes owe their discovery to their role as oncogenes in mouse mammary tumors. The goal of this grant, now at its end, has been to understand more about the function of the *Wnt* signaling molecules in cancer and in normal development (Nusse and Varmus, 1982; Nusse and Varmus, 1992). The emphasis was on finding a receptor for *Wnt* proteins but we were also interested in other genes in the *Wnt* pathway. Problems with working with *Wnt* proteins in vitro had precluded the isolation and characterization of *Wnt* receptors.

Our approach to identify a *Wnt* receptor was two-fold. The *Wnt* gene family includes a *Drosophila* gene *wingless* (*wg*), which genetically has been well-characterized (Klingensmith and Nusse, 1994). In *Drosophila*, we performed genetic screens for modifiers of a *wg* phenotype and we cloned mutant genes. Simultaneously, we used an assay for soluble *wg* protein and an *in vitro* cell culture assay to identify *wg* receptors.

The work was done along the following lines of investigation, as specified in the "statement of work". This report will have the same structure as the statement of work.

(6) Body of text

I To identify genes in *Drosophila* interacting with the *Wnt* gene *wg*

In our initial proposal, we proposed to identify components of *wg* signal transduction in *Drosophila*, by taking advantage of the genetic tools developed in this organism (Klingensmith and Nusse, 1994). By performing genetic screens for suppressors of a *wg*-caused phenotype in the fly, we hoped to uncover mutations in genes that are essential to generate this phenotype. Those genes could encode components of the *wg* signaling pathway. To apply this approach to *Wg* signalling, an adult *wg* phenotype is required. We found that expression of *Wg* in the pupal eye disc via the sevenless promoter, specifically blocks the formation of interommatidial bristles (Cadigan and Nusse, 1996). No other detectable abnormalities were found in either pupal or adult transgenic eyes. *Wg* was shown to act at the level of the proneural genes to block the formation of the sensory organ precursor cells that give rise to the interommatidial bristles.

Over the past years, we have continually performed a genetic screen that allowed not only for the identification of dominant suppressors on the autosomes that modified the adult *Wg* phenotype in the eye, but also allowed for the identification of dominant enhancers (Brunner et al., 1997). Instead of the normal *Wg* cDNA, the temperature sensitive allele of *wg* (wg^{IL114}) (van den Heuvel et al., 1993) was used (P[sew-Wg^{IL114}]). Growth of these transgenic flies at the intermediate temperature resulted in a sensitized background in which only half the number of interommatidial bristles is lacking. Transgenic eyes have a temperature sensitive reduction in the number of interommatidial bristles. At the permissive temperature of 22 ^{o}C, interommatidial bristles are normally formed. At 16 ^{o}C, the restrictive temperature, a strong reduction in the number of interommatidial bristles is observed. These transgenic flies were crossed to ethyl methane sulfonate (EMS) mutagenized males and the F1, which was

grown at an intermediate temperature of 17.6 ^{o}C, was screened for either an enhancement or a suppression of the amount of bristles in the eye. In this way, a lethal complementation group consisting of two dominant enhancers was identified on the third chromosome that we named ***tartaruga* (*trt*)**. This complementation group was further analyzed for interactions with *wg* outside of the eye. In order to find out whether *trt* modifies *wg* activity during embryogenesis as well, the embryonic cuticle phenotype was analyzed of germline clone embryos. Germline clone embryos are embryos that lack any Trt wildtype product. They are derived from female germline mosaics that are homozygous mutant for *trt*. In these germline mosaic clones the maternal contribution of the nurse cells to the oocyte consists only of mutant Trt RNA. Germline mosaics were made with the autosomal FLP-DFS technique. The cuticle phenotype of these germline clone embryos was entirely naked, with only occasional denticles, which is in agreement with the enhancing effect of *trt* on *wg* activity. This was the first evidence that *trt* enhances *wg* activity outside of the eye as well.
The naked cuticle phenotype was seen in 33% of the embryos. The remaining 67% did not have any cuticle and did not show to have undergone any development at all (result not shown). The germline mosaics that give rise to these embryos were made using the trt^{10A20} allele. Germline clone mosaics using $trt^{7A5\text{-}B}$ did not result in the production of any eggs. This may be due to the requirement of *trt* in oogenesis or to the effect of another mutation on the $trt^{7A5\text{-}B}$ chromosome.

In order to establish whether the En domain in these germline clone embryos was broadened (Bejsovec and Martinez-Arias, 1991), *Wg* and En antibody stainings were performed. In *trt* germline clone embryos, we indeed noticed an expansion of the En domain, reminiscent of that seen in HS-*Wg* embryos. In addition, there was also an expansion of the *Wg* domain. This is an indication that *trt* causes a de-repression of *Wg* expression itself. *Wg* and En expressing cells covered the whole parasegment (Bejsovec and Martinez-Arias, 1991). In older germline clone embryos, a large variety in *Wg* and En expression patterns was observed. Invariably, the broadening of the En domain was more prominent to the posterior of the embryo. This phenotype was not rescuable by the presence of paternal wildtype gene product. Both zygotic homozygous and zygotic

heterozygous germline clone embryos revealed the same *Wg* and En expression patterns, indicating that this phenotype is strictly caused by the lack of maternal wildtype Trt RNA.

Trt derepresses *Wg* at the transcriptional level

In order to test whether *trt* de-represses *Wg* itself, mosaic clones of *trt* were generated in imaginal discs which were subsequently stained with a rabbit *Wg* antibody. These mosaic clones were generated with the FLP-FRT system reported by (Xu and Rubin, 1993). For this purpose, the trt^{10A20} allele was recombined on a FRT chromosome and crossed to flies containing the myc-FRT chromosome. Recombination on the FRT elements was induced in flies containing both FRT chromosomes by activation of the flipase (FLP) gene through heatshocking 24-48 hrs or 48-72 hrs after egg laying (AEL). In this way, cells were produced that were either homozygous mutant for *trt* or that contained two copies of the myc gene. Staining with a myc antibody allowed for the localization of the mosaic clone and for the localization of the corresponding twin spot. Mosaic clones of *trt* in wing and leg imaginal discs as well as in the haltere and antennal discs indeed showed high ectopic expression of *Wg* protein. However, only clones that were localized close to the endogenous *Wg* domain exhibited this ectopic expression. The mosaic clones that are homozygous mutant for *trt* were slightly larger and rounder than the corresponding twin spots.

Mosaic *trt* clones in adult wildtype eyes, wings and legs

Mosaic clones of trt^{10A20} cells in the adult wildtype eye were also generated with the FLP-FRT system. In this experiment, the trt^{10A20} -FRT chromosome was recombined with the w^{+}-FRT chromosome at 24-48 hrs AEL giving rise to homozygous *trt* mutant cells that had lost the w^{+} eye marker. A mosaic *trt* clone lacked bristles and caused an irregularity in ommatidial structure. Closer analysis revealed that the lack of

interommatidial bristles was a cell autonomous effect and therefore not attributable to the de-repression of a diffusible protein such as *Wg*.

Mosaic *trt* clones were also analyzed in wings and legs. In order to be able to detect these clones the y^+ body color was used as marker. Recombination was allowed to take place at different times in development between the trt^{10A20}-FRT chromosome and the y^+-FRT chromosome. Clones of cells homozygous for trt^{10A20} had lost the y^+ marker and were yellow in color. Upon heatshocking 24-48 hrs AEL, wings were shown to be completely misfolded and blistered (not shown). Furthermore, tumor-like outgrowths were observed especially at the distal tip of the wing. These outgrowths consisted of cells homozygous for trt^{10A20}. Mosaic clones were also generated later in development, at 48-72 hrs AEL, resulting in smaller clones that were less disruptive and therefore easier to analyze. These clones consisted of clumps of cells that were growing between the wing blades and had a disorganized pattern. The tumor-like growths were observed only within the *trt* mutant mosaic clones and were therefore cell autonomous. Furthermore, these tumor-like phenotypes were observed throughout the wing, including sites in which *trt* mutant clones were shown not to induce ectopic *Wg* expression.

Conclusion. By performing a genetic screen in *Drosophila*, we have identified at least one novel gene that is implicated in *wg* function.

II To determine where in the *wg* pathway these genes act and to clone them in molecular form.

Three lethal transposon insertion lines, P(01814), P(j4A5), and P(rN672) do not complement trt^{10A20}. These mapped to 67E-5-7. The first two P lines did not complement $trt^{7A5\text{-}B}$ either, but P(rN672) did. These three P lines did not complement

each other. In 5-10% of the embryos from these P stocks a naked cuticle was observed. To provide evidence that the transposons disrupt *trt* function, they were excised in the presence of recombinase. This resulted in flies that complemented trt^{10A20}. Furthermore, this experiment led to a few imprecise excisions of the transposon elements, which resulted in the generation of four new alleles, j4A5-16.1. j4A5-18.1, rN672-85.1, and 01814-46.2.

The finding of these P lines was the starting point for the cloning of the *trt* gene. The plasmids of these P lines were rescued and the flanking genomic DNA was mapped by means of restriction enzyme analysis. The flanking genomic DNA from P(j4A5) and that from P(rN672) detected two transcripts on this Northern blot, a faint 6.3 kb and a stronger 4.7 kb band. The signals were only seen after a three day exposure suggesting a very low abundance of the transcript. The flanking genomic DNA that detected these transcripts was used to screen a cDNA library from a 0-5 hr embryo collection of wildtype embryos. In this way, a 3.5 kb cDNA was isolated , which appeared to be identical to *Drosophila* EST LD18074 and to include LD18441. This cDNA contained a 5' UTR of 706 nucleotides, an open reading frame (orf) of 2751 nucleotides and a 3' UTR of 180 nucleotides.

The open reading frame (ORF) encoded a 917 amino acid protein. In vitro transcription and translation of the cDNA produced a protein of approximately 100 kDa which corresponds to the predicted size of 96 kDa. Searching the EST database, we found homologous human, mouse, and C. elegans EST clones. Alignment of the partial cDNA clones with the *Drosophila* sequence shows a highly conserved region of 56% similarity. Within this region, there is a zinc finger motif which is similar to the zinc fingers of GATA transcription factors. This protein has significant sequence homology to a protein present in a complex with histone-deacetylases (Wade et al., 1999), indicating that the Tartaruga protein is involved in transcriptional repression.

To proof that this cDNA is indeed encoding the Trt protein, transformant lines were generated that carry the UAS-LD18074 construct. Trt^{10A20}/TM6,Tb was crossed to $trt^{7A5\text{-}B}$/TM6,Tb in the presence of the presumptive Trt transgene which was driven by arm-GAL4 and grown at 18 ^{o}C. As control, the same cross was performed in

the absence of the *trt* transgene. In this way, rescue was shown until puparium formation. trt^{10A20}/ $trt^{7A5\text{-}B}$ is embryonic lethal except for a percentage of 3% of escapers that develop until puparial stage. By means of the Chi^2 test, however, we were able to repeatedly show that in the presence of the Trt transgene, a significantly higher amount of embryos hatch and develop until puparial stage.

Conclusion: We have indeed cloned the cDNA encoding the Trt protein, which we now know to be a member of a complex also containing a histone deactylase enzyme.

III. To assay whether *wg* protein binds to putative receptor molecules.

Frizzled proteins are cell surface *Wnt* receptors characterized by an extracellular cysteine rich domain (CRD) followed by seven transmembrane segments((Vinson et al., 1989). Members of both protein families have been conserved in species as evolutionarily distant as nematodes and (Wang et al., 1996). The prototypic member of the Frizzled family is the *Drosophila* tissue-polarity gene *frizzled* (*fz*), which was identified because of its phenotype in the adult cuticle (Adler, 1992). Null alleles of *fz* are viable as adults but their bristles, hairs and ommatidia lose their characteristic polarities (Adler, 1992), a characteristic that is referred to as a tissue or planar polarity phenotype. Historically, tissue polarity signaling has been studied without reference to *Wnt* signaling, although both signaling pathways are known to be affected by mutations in the *dishevelled* gene (Krasnow et al., 1995). A second member of the Frizzled family in *Drosophila*, *Drosophila frizzled 2* (*Dfz2*), was identified by sequence homology (Bhanot et al., 1996). Both *fz* and *Dfz2* are expressed during embryonic and larval life (Bhanot et al., 1996), with fz expression in the embryo also being maternally derived. Mutations affecting only the *Dfz2* gene have not been described (Kennerdell and Carthew, 1998).

Two years ago, we reported our findings on the identification of a receptor for the *Wg* protein. We found that *Drosophila* S2 cells do not respond to the *wg* protein, indicating that they lack one or more components of *Wg* signaling. We tested whether transfection of receptor candidates would make S2 cells responsive to the *wg* protein. D*fz*2 is expressed in a *Drosophila* clone-8 cell line that is *wg*-responsive, but not in a non-responding S2 cells (the assay for *wg* activity being the stabilization and subsequent accumulation of the Arm; (Van Leeuwen et al., 1994). After transfection with the D*fz*2 gene, S2 cells are able to transduce the *wg* signal. In addition, the S2 cells can now bind *wg* protein on their cell surface [Bhanot, 1996 #24].

Hence, we have shown that the *Dfz-2* gene fulfills two criteria to be a receptor for the *Wg* protein: *Wg* binds to the *Dfz-2* and binding leads to a biological response; an increase in intracellular Arm concentration. In most vertebrates, more than 10 *Wnt* genes have been identified. As expected, there exists indeed a large family of fz-like genes in vertebrates, likely candidates for receptors for the other *Wnt* proteins [Wang, 1996 #353].

To examine whether Dfz2 functions as a receptor for *Wg* in vivo, we created transgenic flies containing UAS binding sites for yeast Gal4 (Brand and Perrimon, 1993), in front of a truncated Dfz2 cDNA predicted to encode the extracellular domain anchored to the cell surface via a glycerol-phosphatidyl inositol linkage. This truncated protein (GPI-Dfz2) binds *wg* protein in cell culture (Bhanot et al. 1996) but should not be able to transduce the signal to intracellular targets, since it lacks the seven transmembrane and intracellular domains. Therefore, if Dfz2 and *wg* can interact in vivo, GPI-Dfz2 should block *wg* signaling by acting as a sponge, binding the protein non-productively. The Gal4 binding sites in the GPI-Dfz2 transgene allow it to be misexpressed in a variety of patterns through the use of Gal4 expressing lines (Brand and Perrimon, 1993). Expression of GPI-Dfz2 in the wing pouch of wing discs abolishes the expression of the *wg* targets ac and Dll. Experiments in the embryo, wing and eye all indicate that GPI-Dfz2 efficiently blocks *wg* signaling in these tissues (Cadigan et al., 1998). While these data do not conclusively demonstrate that the Dfz2 locus is

required for *wg* signaling, they are consistent with that hypothesis and experiments described below strengthen this view.

Wg induces several bristle types at or adjacent to the margin and loss of *Wg* signaling in clones results in a lack of these bristles and notches in the wing blade. A similar notched phenotype is observed in animals containing 71BGal4, which is active in the wing blade primordium and a weak UAS-GPI-Dfz2 transgene. With a strong UAS-GPI-Dfz2 line, up to one third of the wing blade is missing. Thus, in addition to previously observed block in *wg* target gene expression in wing discs (Cadigan et al., 1998), GPI-Dfz2 does result in penetrant defects in the adult wing consistent with blocking *Wg* action.

To strengthened this connection between GPI-Dfz2 and *Wg* signaling, we examined whether mutations in *wg* or pathway components could modify the wing phenotype. Because of the cold sensitivity of Gal4, these animals showed only occasion notches. However, animals at 25°C always displayed notches when heterozygous for wg^{IN}. Similar genetic interactions were also observed with another *wg* allele (wg^{CX4}) and alleles of *dsh*, *porc* and *arm* ,which are required for *Wg* signaling (reviewed in (Cadigan and Nusse, 1997). Thus GPI-Dfz2 acts antagonistically with *Wg* signaling at the genetic level.

If GPI-Dfz2 blocks *Wg* signaling by competing with the endogenous receptor for *Wg* binding, then it should have no effect on phenotypes generated by signaling components downstream of *wg*. Activation of the pathway in an *wg*-independent manner has been achieved by overexpression of *dishevelled* (*dsh*; Cadigan and Nusse, 1996). In the eye, expression of *wg* and *dsh* using glass (GMR)-Gal4 resulted in a much smaller, glassy eye phenotype. The smaller eye is not due to a direct affect on morphogenetic furrow progression not surprising since GMR-Gal4 is only active behind the advancing furrow. Co-expression of GPI-Dfz2 almost completely blocks the GMR-Gal4/UAS-*wg* phenotype but has only a subtle affect on UAS-dsh.

To test whether Dfz2 expression is important for normal wing development, we created transgenic UAS-Dfz2 flies to allow misexpression of Dfz2. All surviving animals have ectopic bristles on their wing similar to ectopic *wg* expression. Consistent with the hairy wing phenotype, IJ3 Gal4/UAS-Dfz2 discs have a dramatic increase in cells expressing high levels of ac and these cells are found at a greater distance from the *wg* stripe than in controls and presumably cause the ectopic bristles seen in adult wings.

Thus, misexpression of Dfz2 at high levels throughout the wing pouch expands the domains of both short and long range *wg* targets.

The increased activation of *wg* targets by misexpression of Dfz2 could be due to a heightened response of the cells to the *wg* signal, or a constitutive activation of the signaling pathway. To address this, we examined the effect of Dfz2 misexpression in wg^{ts} discs where *wg* activity was blocked. Both ac and Dll expression was dramatically reduced under these conditions, to levels seen in wg^{ts} discs under the same conditions in an otherwise wild type background. This indicates that the primary effect of Dfz2 misexpression is to potentiate the ability of *wg* to signal to target cells.

Misexpression of Dfz2 or GPI-Dfz2 causes a dramatic post-transcriptional spread of *wg* protein with IJ3 Gal4/UAS-Dfz2 discs having high levels of *wg* several cells away from the RNA stripe. The ectopic *wg* protein is found on what appears to be the surface of the cells, and in contrast to endogenous *wg*, predominately basal laterally.

In conclusion, expression of the extracellular domain of Dfz2 efficiently blocks *wg* signaling (Cadigan et al. 1998), suggesting binding in vivo. In contrast to *Dfz2*, misexpression of *frizzled* in the developing wing has no affect on *Wg* signaling or *Wg* protein distribution. Conversely, misexpression of *fz* severely disrupted planar polarity in the wing and eye but *Dfz2* did not. These data argue for a model where Dfz2 and Fz have distinct signaling activities, the former for *Wg* signaling and the latter for planar polarity. These data support the view that Dfz2 is a specific receptor for *wg* in vivo. Together with our

earlier data, we have therefore achieved much of our working goals of this grant.

Deletion of the *Dfz2* locus and construction of *fz, Dfz2* double mutants

In earlier experiments we demonstrated that both Dfz2 and Fz are able to bind *Wg* in cell culture assays and transduce the *Wg* signal (Bhanot et al., 1996). However these experiments did not address the role of Fz and Dfz2 in the intact organism or the question of specificity of ligand-receptor interactions *in vivo*.

In the present study, we have generated a small deficiency which encompasses the *Dfz2* locus. We show that embryonic development in general, and *Wg* signaling in particular, is nearly normal in deletion homozygotes. However, embryos lacking both maternal *fz* and zygotic *fz* and *Dfz2* display defects in epidermal patterning, RP2 neuron specification, midgut morphogenesis, and heart formation, which are extremely similar to the defects exhibited by *wg* mutants.

To identify a P-element integrated within or near the *Dfz2* locus, we characterized five *Drosophila* lines in which a P-element had been reported to map in or near 76A. Genomic DNA flanking each P-element insertion site was cloned by plasmid rescue and hybridized against a panel of P1 clones carrying the *Dfz2* locus. The insertion site of a single P-element line, 469, was found to reside on a subset of the P1 clones, and further mapping and sequencing showed that the 469 P-element is inserted 60 bases upstream of *Dfz2* exon 1. Despite the proximity of the 469 P-element to the *Dfz2* transcription unit, *Dfz2* transcripts from 469 embryos are indistinguishable from the wild type in size and abundance, and flies homozygous for this insertion show no obvious morphologic defects during embryogenesis or adulthood.

The 469 line was used in a gamma-ray mutagenesis screen to generate deficiencies in the *Dfz2* locus, and one mutant line, 469-2, was found to harbor a deletion that encompasses only the 76A region as judged by cytological mapping of its polytene chromosomes. For convenience we will simply refer to the 469-2 deletion hereafter as Df(3L)*Dfz2* or as a *Dfz2* deficiency. Our initial assessment of mutant phenotype focused on patterning in the embryonic cuticle. In wild-type larvae, the

cuticle is covered by an alternating pattern of denticle belts and naked cuticle which is specified by the interaction of segment polarity genes in the embryonic epidermis. *Wg* action specifies both denticle diversity in the anterior half of each segment and naked cuticle in the posterior half. In *wg* mutants, there is loss of naked cuticle characteristic of the posterior half and reduction in the diversity of denticles covering the anterior half of each segment such that the denticles present on the cuticle are morphologically similar to those of wild-type row 5.

469-2 homozygotes die shortly after hatching and exhibit a subtle disorganization of denticle patterning with occasional ectopic denticles in posterior compartments. These data suggest that *Dfz2* and/or other genes removed by the 469-2 deficiency play a minor or largely redundant role in cuticle patterning during embryogenesis. In light of the similarity in sequence and *Wg*-binding properties exhibited by Fz and Dfz2 we tested the hypothesis that Fz and Dfz2 might function redundantly by constructing double mutants in which various *fz* alleles (at map position 70D6) were recombined onto the 469-2 deficiency chromosome. For these experiments, and in those described below, we have used the following *fz* alleles: (1) fz^{P21}, a frameshift near the amino terminus which behaves as a null mutation; (2) fz^{R52}, a premature termination codon that removes the last transmembrane domain and which behaves as a null mutation with respect to the adult tissue polarity phenotype, but which behaves as a hypomorph in the embryo experiments described below; and (3) fz^{D21}, a deficiency.

While embryos homozygous for the *Dfz2* deficiency have essentially normal segmentation, *Dfz2* deficiency embryos in which both *fz* alleles are also mutant display variable segmentation defects. The defects range from a few extra denticles in the posterior part of some segments to a complete replacement of naked cuticle with denticles on the ventral side of the embryo. This "lawn of denticles" is reminiscent of phenotypes obtained with a temperature sensitive allele of *wg* (Bejsovec and Martinez-Arias, 1991) or mutations in DTCF, the DNA-binding protein that mediates *wg* signaling in the nucleus (Brunner et al., 1997; Van de Wetering et al., 1997). Thus, the absence of zygotic *fz* and *Dfz2* produces a cuticle phenotype consistent with a partial defect in *Wg* signaling.

fz and *Dfz2* are required during embryogenesis to maintain *en* and *wg* expression in the epidermis

In the wild-type epidermis, *wg* functions in an autocrine pathway to maintain its own expression and in a paracrine regulatory loop to maintain expression of *en* in adjacent cells. In the epidermis at gastrulation, when *wg* function is first detected, a stripe of cells in the anterior half of each parasegment expresses *wg* and an adjacent stripe of cells in the posterior half express *en* . This pattern is initiated by pair-rule and gap genes, but its maintenance requires paracrine signaling by *Wg* to the *en* expressing cells and both paracrine signaling by Hh and autocrine signaling by *Wg* to the *wg* expressing cells. Thus, in *wg* mutant embryos the pattern of *wg* and *en* gene expression is initiated correctly but is not maintained. In *fz, Dfz2* double mutant embryos, *en* and *wg* are initially expressed in the epidermis in the correct pattern, but the abundance of both proteins declines significantly. By contrast, within the CNS *en* expression is maintained. The effect on *en* and *wg* expression in the epidermis is less severe in *fz, Dfz2* double mutant embryos heterozygous for fz^{R52} / fz^{D21} compared to embryos carrying fz^{D21} / fz^{P21}.

In conclusion, the experiments reported here indicate that *in vivo* there are overlapping interactions between Frizzled and *Wnt* family members such that a single *Wnt* can interact with more than one Frizzled receptor. We infer that the converse is also true based on the available data regarding Fz function in the embryo and imaginal disc. Presumably, the promiscuous binding observed between various *Wnt* and Frizzled family members in cell culture experiments represents the *in vitro* correlate of the broad *in vivo* specificities inferred from genetic experiments. The nearly complete redundancy of Fz and Dfz2 explains why *Wg* receptors were not identified in earlier genetic screens for mutants defective in embryonic patterning (Nüsslein-Volhard and Wieschaus, 1980). The ability of maternal *fz* mRNA and/or Fz protein to fully

rescue the zygotic loss of both *fz* and *Dfz2* would also have precluded identifying these genes in a screen for zygotic patterning mutations.

IV. To clone mammalian homologs of receptor genes.

In the original proposal, we intended to clone human and mouse receptor genes, after identifying the *Drosophila* receptor. However, when we identified Frizzleds as receptors for *Wnt* proteins, most of the mammalian Frizzled homologs had already been found. Together with Dr. Uta Francke (HHMI, Beckman Center, Stanford) we were involved in cloning one additional member of the human Frizzled gene family, FZD3. This gene is present in a region of human chromosome 7 that is implicated in Williams Syndrome (WS).

Cloning and sequence analysis of FZD3

A chromosome 7 specific cosmid library was screened with inter-ALU PCR products of YAC clones from within the common WS deletion region Positive cosmids were confirmed by STS content mapping, including the markers D7S489, D7S613, D7S1870, D7S2472 and ELN.Cosmid ends were sequenced and analyzed by PCR, with respect to presence in the deletion, with somatic cell hybrids containing either the 7q11.23 deleted or the normal chromosome 7 from a proband with typical WS. A modified CpG island cloning method was used to search for coding sequences in the five cosmids that mapped within the deletion region. Out of the 30 clones sequenced, one clone contained a 700 bp insert that, by database comparison, showed homology to several frizzled family members. This clone, called fzd3, was mapped to cosmids 082 and 1124 that are both positive for the marker D7S489B. D7S489B is deleted in all of the probands who have deletions of ELN and is the most centromeric marker in the common deletion region. A fetal brain cDNA library (Stratagene) was screened and a 4.4 kb clone obtained. This clone was found to be chimeric: 2.2 kb of the clone was mapped to chromosome 6 by typing a somatic cell hybrid mapping panel.

The transcript, called FZD3, encodes a 591 amino-acid open reading frame (ORF) with high homology to members of the frizzled family. Although we did not isolate a full-length cDNA clone, we believe we have identified the complete ORF as there was no other methionine codon between the initiator ATG and stop codons in the 5' untranslated region. A Kyte-Doolittle hydropathic profile suggests that the predicted protein contains seven transmembrane domains. In addition, there is a large cysteine-rich region in the N terminal part. Both are common structural motifs found in the frizzled family. When compared to the G protein-coupled seven transmembrane receptor superfamily, FZD3 and other frizzled family members retain some of the features conserved among that superfamily, including N-linked glycosylation sites (Asn-X-Ser/Thr) in the extracellular domain (Asn53 and Asn158) and two cysteine residues (Cys294 and Cys388) in the second and the third extracellular loops. The two highly conserved cysteine residues have been proposed to play a role in maintaining the active conformation of the receptor. FZD3 lacks the other conserved features occurring in G protein-coupled receptors, including a palmitylated cysteine residue in the C terminal region that is thought to be involved in activating G proteins.

The expression pattern of FZD3 is distinctive. Northern analysis using the 2.2 kb cDNA probe revealed a single 2.4 kb transcript expressed in skeletal muscle, brain and testis. On longer exposures of the autorad a transcript was also seen in pancreas but not in liver and kidney as both these lanes were relatively underloaded. FZD3 is also moderately expressed in most of the endocrine tissues, including pancreas, thyroid, adrenal cortex as well as in small intestine and stomach. Variably-sized weak transcripts were detected in testis (3.5 kb), thyroid (4.4 kb) and pancreas (8 kb). These could be transcripts of related genes. On a Northern blot exposed for only 3 h, the 2.4 kb FZD3 mRNA appears to be equally expressed in all parts of the brain

To determine whether FZD3 binds the *Wnt* protein *Wg*, we used the same *Wg* binding assay on *Drosophila* S2 cells and human 293 cells transfected with FZD3 expression constructs. FZD3, under the control of a metallothionein promoter, was stably transfected into *Drosophila* S2 cells that do not produce *Wg* protein. Expression of FZD3 was induced by growing the cells overnight in the presence of copper sulfate. Cells were then incubated with conditioned medium from *Wg*-producing S2 cells and

subsequently with affinity-purified polyclonal antibodies to *Wg*. The S2 cells expressing FZD3 show surface staining, while the S2 control cells not expressing FZD3 show randomly scattered background spots.

We conclude from these experiments that stably and transiently expressed human FZD3 receptors bind *Wg* and that FZD3 is a bona fide human Wnt receptor.

(7) Key Research Accomplishments

All major goals of the grant have been achieved:

- We have identified a novel gene in Wg signaling, tartaruga
- We have cloned the tartaruga gene and showed that it is conserved in evolution
- We have found that Frizzled proteins are receptors for Wnts
- We have demonstrated that Dfz2 is genetically required for Wg signaling in Drosophila, when the related Fz gene is also removed genetically
- We have contributed to cloning one novel human Frizzled gene

(8) Reportable outcomes

Manuscripts (all attached as appendices, see (11)

Bhanot, P., Brink, M., Harryman Samos, C., Hsieh, J. C., Wang, Y. S., Macke, J. P., Andrew, D., Nathans, J. and Nusse, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature *382,* 225-230.

Cadigan, K., Fish, M., Rulifson, E. and Nusse, R. (1998). Wingless repression of Drosophila frizzled2 expression shapes the wingless morphogen gradient in the wing. Cell *93,* 767-777.

Cadigan, K. and Nusse, R. (1996). wingless signaling in the Drosophila eye and embryonic epidermis. Development *122,* 2801 - 2812.

Cadigan, K. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Genes & Dev. *11,* 3286-3305.

Nusse, R., Samos, C. H., Brink, M., Willert, K., Cadigan, K. M., Wodarz, A., Fish, M. and Rulifson, E. (1997). Cell culture and whole animal approaches to understanding signaling by Wnt proteins in Drosophila. Cold Spring Harbor Symp. Quant. Biol. *LXII,* 185-190.

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Funding Applied for

1. A novel gene with a potential tumor suppressor phenotype.
   R. Nusse PI
   USAMRMC IDEA award, BCRP Proposal BC980295, was awarded to start in 1999

2. 1R01GM/CA60388-01, Signaling by Frizzled Proteins in Drosophila
   R. Nusse, PI.
   Pending with the NIH

3. Identification of specific mutations that activate a growth factor receptor, using a novel and generally applicable method.
   R. Nusse, PI
   USAMRMC IDEA award, BCRP Proposal BC990566
   Pending

(9) Conclusions

The findings we made on the Frizzled molecules as receptors for Wnts are an important breakthrough in our understanding of Wnt signaling. This discovery was hailed in Science Magazine as one of the 10 most important scientific findings in the year 1997 (Science Vol 274, page 1988, 1997) and has been the subject of many short reviews in other journals. As this was the goal of the proposal, we feel that we have done our work quite well. In addition, the identification of the tartaruga gene as a repressor of wingless signaling in Drosophila and the homology between this gene and components of the histone-deacetylase complex is a major contribution.

We expect that these findings are not only going to be important for basic research but also for the treatment of cancer, including breast cancer. Specific cell surface receptors, such as the Frizzleds, are important targets for therapeutic intervention. Moreover, point mutations in those receptors may occur in human cells and lead to abnormal growth.

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(11) Appendices

(13) List of Personnel receiving pay

Cadigan, Ken
Harris, Frank
Jou, Austin
Kon, Charlene
Kozopas, Karen
Le, Dominic
Lessing, Derek
Logan, Catriona
Mayo, Ramon
Moreno, Maria
Smith, Wayne
Stone, Joyce
Willert, Karl
Wu, Chi-Hwa
Xu, Yu
Zakrajsek, Irena

A new member of the *frizzled* family from *Drosophila* functions as a Wingless receptor

Purnima Bhanot*, Marcel Brink†, Cindy Harryman Samos†, Jen-Chih Hsieh*‡, Yanshu Wang*‡, Jennifer P. Macke*‡, Deborah Andrew§, Jeremy Nathans*‡‖ & Roel Nusse†

* Department of Molecular Biology and Genetics, § Department of Cell Biology and Anatomy, ‖ Department of Neuroscience, and ‡ Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Room 805 PCTB, Baltimore, Maryland 21205, USA
† Howard Hughes Medical Institute and Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305, USA

Receptors for Wingless and other signalling molecules of the *Wnt* gene family have yet to be identified. We show here that cultured *Drosophila* cells transfected with a novel member of the *frizzled* gene family in *Drosophila*, *Dfz2*, respond to added Wingless protein by elevating the level of the Armadillo protein. Moreover, Wingless binds to *Drosophila* or human cells expressing *Dfz2*. These data demonstrate that Dfz2 functions as a Wingless receptor, and they imply, in general, that Frizzled proteins are receptors for the Wnt signalling molecules.

There is abundant evidence that secreted Wnt proteins have important signalling functions during animal development. For example, Wnt proteins have been implicated in cell-lineage decisions in *Caenorhabditis elegans*, in embryonic and adult pattern formation in *Drosophila*, in axis formation and dorsal–ventral polarity determination in *Xenopus* embryos, and in central nervous system (CNS) development and oncogenesis in mice[1–3]. However, the Wnt proteins have been difficult to obtain in a soluble form, a problem that has hampered the development of biochemical and cell biological assays. Most information about the mechanism of Wnt signalling has come instead from the genetic analysis of *Drosophila* segment polarity and the role of the *Wnt* gene *wingless* (*wg*; refs 2,4–8). Within each embryonic segment, production of the *wingless* protein (Wg) by a narrow stripe of cells maintains *engrailed* expression in an adjacent stripe of cells.

In the embryonic epidermis the *wg* signalling pathway is defined by several genes: *dishevelled* (*dsh*)[9,10]; *zeste white* 3 (*zw*3 or *shaggy*); and *armadillo* (*arm*), a member of the beta-catenin gene family[11], which is thought to be inactivated by *zw*3. The *wg* signal seems to counteract the inhibitory effect of *zw*3, leading to activation of *arm*[12–14]. In *Drosophila* embryos the cytoplasmic levels of the *arm* protein (Arm) are increased as a consequence of *wg* signalling[15]. As judged by sequence data, none of the proteins identified thus far in the signalling pathway is a Wg receptor.

On the basis of genetic interactions between *wg* and other genes in the *wg* pathway, we have established a tissue-culture system for *wg* signalling[16]. In this assay, Wg produced by *Drosophila* S2 cells is added in soluble form to a cell line (clone 8) derived from *Drosophila* imaginal discs[17]. Like *Drosophila* embryos, clone 8 cells respond to Wg by specifically increasing the levels of hypophosphorylated Arm[16], suggesting that these cells express a receptor specific for Wg.

Here we report the identification of a novel *Drosophila* gene, *frizzled*2 (*Dfz*2), and demonstrate that it functions as a Wg receptor in cultured cells. *Dfz*2 was identified in the course of characterizing a large family of vertebrate and invertebrate homologues of the *Drosophila* gene *frizzled* (*fz*)[18]. Mutations in *fz* result in aberrant orientations of adult cuticular structures, a tissue polarity phenotype[19–21]. The *fz* sequence predicts an encoded protein with an amino-terminal cysteine-rich extracellular domain followed by seven transmembrane segments[22,23]. These characteristics have led to the suggestion that *fz* is a receptor for an unidentified ligand that transmits tissue-polarity information[24]. Although *wg* does not seem to be involved in the tissue-polarity pathway and *fz* does not seem to be involved in the segment-polarity pathway, a possible link between the two pathways is suggested by the requirement in each for *dsh* function[10,25].

Molecular cloning of *Dfz2*

Using the sequences of *fz* and three mammalian *fz* homologues, degenerate polymerase chain reaction (PCR) primers were designed for the purpose of amplifying additional *fz*-like sequences[18]. PCR amplification using *Drosophila* genomic DNA as a target revealed a novel *frizzled* family member, *Drosophila frizzled*2(*Dfz*2). Isolation and sequence analysis of genomic and complementary DNA clones corresponding to *Dfz*2 revealed a single coding exon containing an open reading frame of 694 amino acids (Fig. 1*a*). The predicted *Dfz*2 protein (*Dfz*2) resembles all other members of the *frizzled* family in having the following structural motifs (beginning at the N terminus): a putative signal sequence, a domain of 120 amino acids with an invariant pattern of ten cysteine residues, a highly divergent region of 40–100 largely hydrophilic amino acids that is predicted to be flexible, and seven putative transmembrane segments (Fig. 1*b*). The C terminus of Dfz2 resembles that of most mammalian frizzled protein in ending with the sequence S/T-X-V. A comparison with all known *frizzled* sequences shows that Dfz2 most closely resembles human fz5 and mouse fz8 with which it shares 49% and 45% amino acid identity, respectively. Fz and Dfz2 share 33% amino-acid identity. The *Dfz*2 gene resides at 76A on the polytene chromosome map as determined by *in situ* hybridization (data not shown).

Developmental expression of *Dfz2*

As a first step in elucidating the function of *Dfz*2 we examined temporal and spatial expression patterns by northern blot analysis, *in situ* hybridization, and immunostaining. A 5.5-kilobase (kb) Dfz2 transcript is found throughout the *Drosophila* life cycle, most prominently during embryogenesis and in late larval and pupal life (Fig. 1*c*). At 2 hours post-fertilization, embryos have low levels of Dfz2 RNA, which is presumably of maternal origin. *Dfz*2 expres-

FIG. 1 Isolation and characterization of *Dfz*2—a novel *frizzled* family member in *Drosophila*. *a*, Alignment of the predicted amino-acid sequences of Dfz2 and Fz. Identical residues are indicated in the consensus and conserved cysteines in the cystein-rich domain[18] are in bold face. *b*, Hydropathy profiles of Dfz2 and Fz proteins were calculated according to ref. 37 with a window size of 15 amino acids; increasing hydrophobicity is downwards. The seven putative transmembrane domains and the signal peptide are indicated by a solid line. *c*, Expression of *Dfz*2 RNA during *Drosophila* development and in clone 8 and S2 cells. Upper panel, a 950-bp segment from the *Dfz*2 coding region was used to probe a blot containing 40 μg RNA per track from the indicated stages of *Drosophila* development or 30 μg total RNA from S2 cells and clone 8 cells. For the embryo samples, times during development are indicated in hours. A 5.5-kb transcript was detected at all stages, including 0–2 h post-fertilization. Lower panel, a ribosomal protein 49 (RP49) probe was used to probe the same blot to control for sample loading and transfer efficiency.

METHODS. *D. melanogaster* genomic DNA was used as a template for PCR amplification using fully degenerate primers corresponding to conserved sequences YPERPI (sense) and WFLAA (antisense) as described[18]. A single PCR product corresponding to codons 348–425 of *Dfz*2 was obtained and used to screen a *D. melanogaster* genomic DNA library in bacteriophage λ. Sequence analysis of cloned genomic DNA revealed an open reading frame of 694 codons with 33% amino-acid identity to Fz. Partial sequences from 6 independent cDNA clones obtained from a 0–9-h embryo cDNA library (gift from K. Zinn) indicate that the open reading frame is contained within a single exon, that there is at least one 5′ non-coding exon, and that the putative initiator methionine is the 5′-most proximal ATG codon and is located 5 codons 3′ of an in-frame termination codon. The Dfz2 and Fz sequences were aligned using GeneWorks software, and hydropathy profiles were calculated using MacVector 3.5 software. RNA from various stages of *Drosophila* development was isolated by the guanidinium–phenol method[38] or from tissue culture cells by LiCl–urea precipitation[39]. RNA was transferred and hybridized as described[38].

a

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Dfz2       MRHNRLKVLI LGLVLLLTSC RADGPLHSAD HGMGGMGMGG HGLDASPAPG     50
fz         MWRQILFILP -TLIQGVQRY -DQSPLDASP YYRSGGGL-- --M---ASSG     41
Consensus  M....L..L. ..L..... ....PL.... ....G.G... .........G     50

Dfz2       YGVPAIPKDP NLRCEEITIP MCRGIGYNMT SFPNEMNHET QDEAGLEVHQ    100
fz         TELDGLPHHN --RCEPITIS ICKNIPYNMT IMPNLIGHTK QEEAGLEVHQ     89
Consensus  ......P... ..RCE.ITI. .C..I.YNMT ..PN...H.. Q.EAGLEVHQ    100

Dfz2       FWPLVEIKCS PDLKFFLCSM YTPICLEDYH KPLPVCRSVC ERARSGCAPI    150
fz         FAPLVKIGCS DDLQLFLCSL YVPVC-TILE RPIPPCRSLC ESAR-VCEKL    137
Consensus  F.PLV.I.CS .DL..FLCS. Y.P.C..... .P.P.CRS.C E.AR..C...    150

Dfz2       MQQYSFEWPE RMACEHLPLH GDPDNLCMEQ PSYTEAGSGG SSGGSGGSGS    200
fz         MKTYNFNWPE NLECSKFPVH GGED-LCVAE ------NTTS SASTAATPTR    180
Consensus  M..Y.F.WPE ...C...P.H G..D.LC... .......... S.........    200

Dfz2       GSGSGGKRKQ GGSGSGGSGA GGSSGSTSTK PCRGRNSKNC QNPQGEKASG    250
fz         SVAKVTTRKH ------QTGV ---------- ---------- ESPH--RNIG    202
Consensus  .......RK. ........G. .......... .......... ..P......G    250

Dfz2       KECSCSCRSP LIFLGKEQLL QQQSQMPMMH HPHHWYMNLT VQRIAGVPNC    300
fz         FVC------P V------QL- --KTPLGMG- -----Y-ELK VG-GKDLHDC    229
Consensus  ..C......P .......QL. .......M.. .....Y..L. V........C    300

Dfz2       GIPCKGPFFS NDEKDFAGLW IALWSGLCFC STLMTLTTFI IDTERFKYPE    350
fz         GAPCHAMFFP ERERTVLRYW VGSWAAVCVA SCLFTVLTFL IDSSRFRYPE    279
Consensus  G.PC...FF. ..E.....W ...W...C.. S.L.T..TF. ID..RF.YPE    350

Dfz2       RPIVFLSACY FMVAVGYLS- ---------R N-FLQNEEIA CDGLL--LRE    387
fz         RAIVFLAVCY LVVGCAYVAG LGAGDSVSCR EPFPPPVKLG RLQMMSTITQ    329
Consensus  R.IVFL..CY ..V...Y... .........R ..F..... ..........    400

Dfz2       SSTGPHSCTL VFLLTYFF-G MASSIWWVIL SFTWFLAAGL KWGNEAITKH    436
fz         GHRQTTSCTV LFM-ALYFCC MAAFAWWSCL AFAWFLAAGL KWGHEAIENK    378
Consensus  ......SCT. .F.....F.. MA...WW..L .F.WFLAAGL KWG.EAI...    450

Dfz2       SQYFHLAAWL IPTVQSVAVL LLSAVDGDPI LGICYVGNLN PDHLKTFVLA    486
fz         SHLFHLVAWA VPALQTISVL ALAKVEGDIL SGVCFVGQLD THSLGAFLIL    428
Consensus  S..FHL.AW. .P..Q...VL .L..V.GD.. .G.C.VG.L. ...L..F...    500

Dfz2       PLFVYLVIGT TFLMAGFVSL FRIRSVIKQQ GGVGAGVKAD KLEKLMIRIG    536
fz         PLCIYLSIGA LFLLAGFISL FRIRTVMKTD G-----KRTD KLERLMLRIG    473
Consensus  PL..YL.IG. .FL.AGF.SL FRIR.V.K.. G........D KLE.LM.RIG    550

Dfz2       IFSVLYTVPA TIVIGCYLYE AAYFEDWI-- ------KALA CPCAQVKGPG    578
fz         FFSGLFILPA VGLLGCLFYE YYNFDEWMIQ WHRDICKPFS IPCPAARAPG    523
Consensus  .FS.L...PA ....GC..YE ...F..W... ......K... .PC.....PG    600

Dfz2       K---KPLYSV LMLKYFMALA VGITSGVWIW SGKTLESWRR FWRRLLGAPD    625
fz         SPEARPIFQI FMVKYLCSML VGVTSSVWLY SSKTMVSWRN FVERLQGKEP    573
Consensus  .....P.... .M.KY..... VG.TS.VW.. S.KT..SWR. F..RL.G...    650

Dfz2       RTGANQALIK QRPPIPHPYA GSGMGMPVGS AAGSLLATPY TQAGGASVAS    675
fz         RT-------- -R---AQAYV ---------- ---------- ----------    581
Consensus  RT........ .R......Y. .......... .......... ..........    700

Dfz2       TSHHHLHHHV LKQPAASHV          694
fz         ---------- ---------          581
Consensus  .......... .........          719
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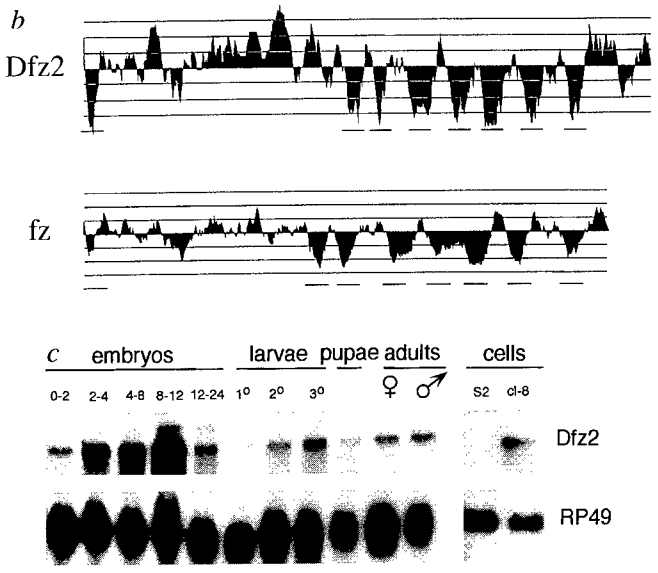


sion is first clearly detected at stage 6, where it is found in all cells between roughly 15% and 70% of egg length, including the invaginating cells of the ventral furrow (Fig. 2*a*). An emerging stripe pattern is evident by early stage 8, and by stage 10 *Dfz*2 expression is clearly seen in 15 stripes in the presumptive head and trunk regions, in the posterior midgut primordium, in a subset of cells at the site of anterior midgut invagination, and in the procephalic lobe (Figs 2*b*, *c*). Beginning at stage 12, during germ-band shortening, *Dfz*2 expression declines in the epidermis and increases in the midgut and visceral mesoderm (Fig. 2*f*). Beginning at stage 9 and continuing throughout embryogenesis, *Dfz*2 expression is also seen in the developing CNS (Fig. 2*d–l*). By stage 17 *Dfz*2 expression becomes limited to the CNS, hindgut and dorsal vessel (Fig. 2*l*).

Transfection of *Dfz*2 in S2 cells

We also measured Dfz2 RNA and protein levels in clone 8 cells, which respond to added Wg as determined by an increase in Arm protein, and in Schneider (S2) cells, which are unresponsive[16,26]. A *Dfz*2 transcript that matches the size of the *in vivo* transcript was observed in clone 8 cells, but no *Dfz*2 transcript was detected in S2 cells (Fig. 1*c*). Similarly, in western blots probed with affinity-purified anti-Dfz2 antibodies, a protein band with an apparent mobility of 65K (and comigrating with Dfz2 produced in transfected S2 cells; see below) was observed in samples derived from clone 8 cells but not S2 cells (data not shown). Transcripts derived from *fz* were not detectable in either S2 or clone-8 cells (data not shown).

It is possible that S2 cells fail to respond to added Wg because they do not express *Dfz*2. We tested this directly by transfecting S2 cells with a *Dfz*2 expression construct. These cells were then

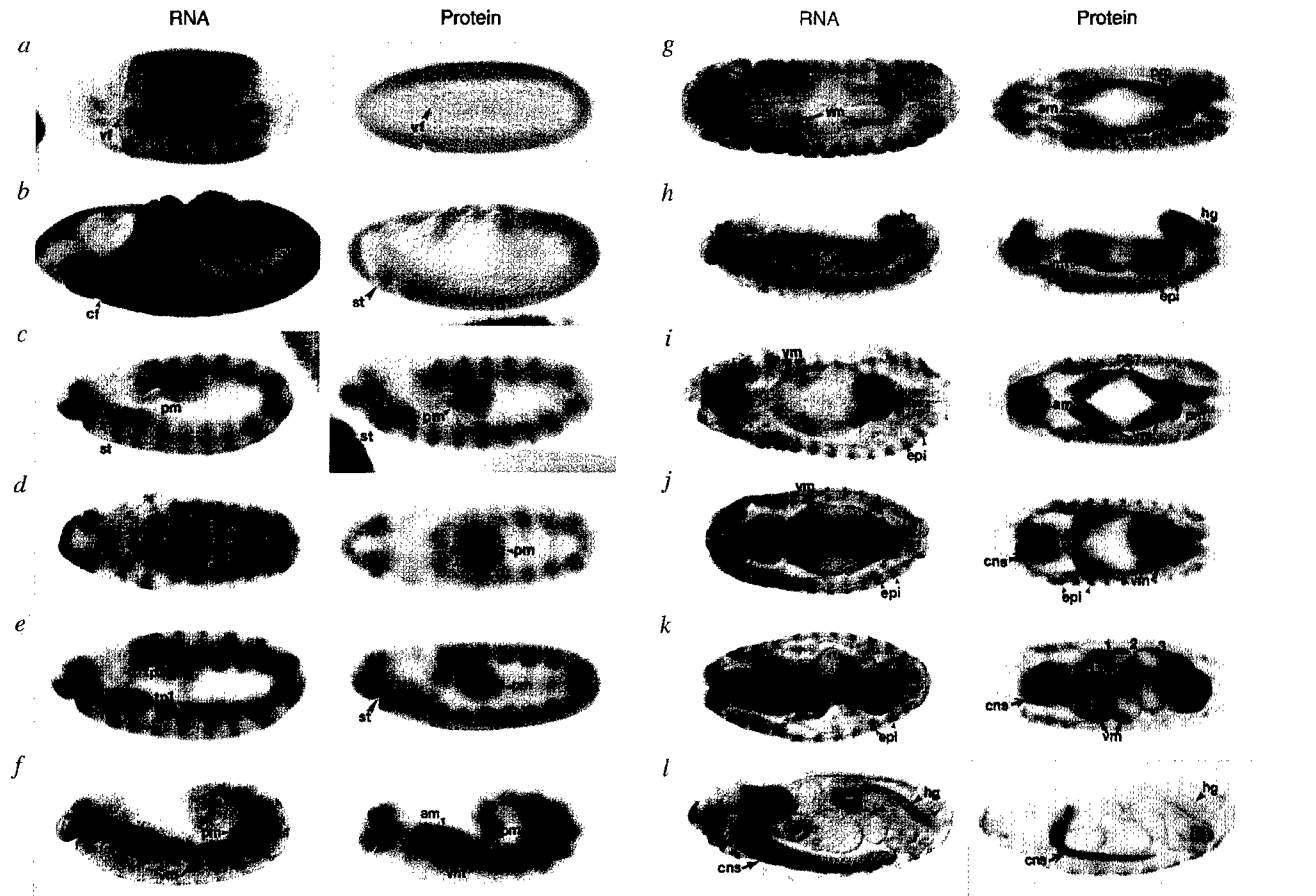


FIG. 2 *Dfz2* RNA and protein in wild-type embryos. Pairs of embryos at the same developmental stage are shown with *in situ* hybridization on the left (blue) and immunostaining on the right (brown). Embryos are oriented with anterior to the left. For embryos shown in a lateral view, dorsal is up. Stages and structures are according to ref. 40. *a*, Stage 6, dorsal/ventral view. *Dfz2* RNA is present in the central region of the embryo and is absent from the anterior and posterior regions. At this stage, Dfz2 protein is below the limit of detection. *b*, Stage 7/8, lateral view. *Dfz2* RNA begins to accumulate in a striped pattern. *c*, Stage 9/10, lateral view. During germ-band extension, *Dfz2* is expressed in 15 stripes in the presumptive head and trunk regions, in the posterior midgut primordium, and in a subset of cells at the site of anterior midgut invagination. *d*, Stage 9/10, dorsal/ventral view. *Dfz2* expression can be seen in the developing CNS. *e*, Stage 10/11, lateral view. *Dfz2* expression resembles stage 9, with transiently higher expression around the primordia of the tracheal pits. *f*, Stage 12, lateral view. During germband retraction, *Dfz2* expression decreases in the epidermis but is maintained at high levels in the anterior and posterior midgut and the presumptive visceral mesoderm. *g*, Stage 13, dorsal/ventral view. The striped pattern of *Dfz2* expression persists in the visceral mesoderm and reappears in cells surrounding the segmental borders. *h*, Stage 13, lateral view. *Dfz2* is expressed at high levels in the hindgut. *i*, Stage 14, dorsal/ventral view. *Dfz2* expression is lower in the anterior and posterior midgut. There is ubiquitous expression in the visceral mesoderm, except in parasegment 7, previously described as the domain of *Ubx* and *dpp* expression[41,42]. *j*, Stage 15, dorsal/ventral view. *Dfz2* is expressed in the CNS, ventral mesoderm, and in cells surrounding the segmental borders. *k*, Stage 16, dorsal/ventral view. *Dfz2* is expressed at high levels in the CNS and in the visceral mesoderm spanning the first midgut constriction and posterior to the second midgut constriction. *l*, Stage 17, lateral view. *Dfz2* expression is primarily in the CNS, with lower levels in the hindgut and the dorsal vessel. Abbreviations: am, anterior midgut; cf, cephalic furrow; cns, central nervous system; epi, epidermis; hg, hindgut; pm, posterior midgut; PS 7, parasegment 7; st, stomodeum; tp, tracheal pit; vf, ventral furrow; vm, visceral mesoderm.

METHODS. Whole-mount embryo *in situ* hybridization was performed on 0–24-h embryos using a digoxygenin-labelled DNA probe encompassing *Dfz2* codons 1–307 as described[43] with minor variations. Fixation was in 4% formaldehyde/1× PBS and the staining reaction was done without levamisole. Identical patterns were obtained with a second probe corresponding to *Dfz2* codons 308–668. Immunochemical localization of Dfz2 protein was done using affinity-purified rabbit antibodies raised against a fusion protein containing the bacteriophage T7 gene-10 protein joined to amino acids 65–314 of Dfz2. Antibodies were purified using a fusion protein containing the *E. coli* maltose-binding protein joined to the same segment of Dfz2. Immunostaining was done as described[44], except that embryos were fixed in Bouin's solution for 30 min instead of 4% formaldehyde/PBS. Antibody staining was visualized by the ABC method (Vector Labs) and embryos were mounted in methyl salicylate.

assayed for the ability to stabilize Arm in response to added Wg. In the absence of Wg, transfected and untransfected S2 cells show similar low levels of Arm irrespective of whether *Dfz2* expression was elevated by copper induction. However, when *Dfz2*-transfected cells were incubated in the presence of Wg, the level of the faster migrating (hypophosphorylated) form of Arm was increased (Fig. 3). This elevation was similar to the response elicited by Wg in clone-8 cells. Increasing *Dfz2* above basal level by copper induction of the metallothionein promoter led to a decrease in Wg responsiveness (Fig. 3), suggesting that at high levels Dfz2 may bind non-productively to second messenger components. Four independent *Dfz2*-transfected cell lines derived from two separate transfections were tested, and all four lines showed Wg-dependent Arm stabilization.

Transfection of *Dfz2* confers Wg binding

The results described above show that *Dfz2* expression confers responsiveness to Wg, consistent with the idea that Dfz2 is a receptor for Wg. To examine Wg binding directly, we incubated *Dfz2*-expressing S2 cells with Wg at 4 °C, and subsequently stained the cells with affinity-purified polyclonal antibodies to Wg. S2 cells expressing *Dfz2* show strong surface staining when incubated with

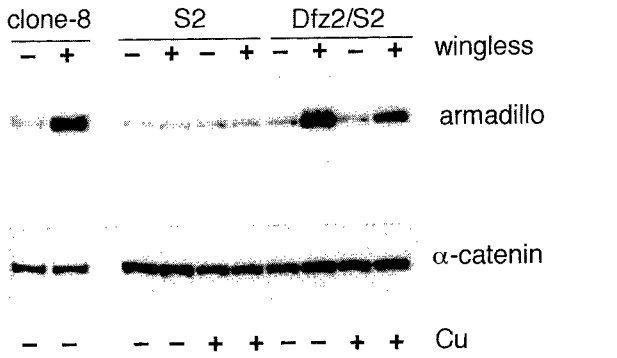


FIG. 3 Addition of soluble Wg leads to an increase in the level of Armadillo protein in *Dfz*2-transfected S2 cells. Clone 8 cells (left), untransfected S2 cells (centre), or *Dfz*2-transfected S2 cells (right) were incubated with concentrated conditioned medium either from S2 cells producing Wg (+Wg) or from control S2 cells (−Wg). Untransfected and *Dfz*2-transfected S2 cells were tested following growth with or without copper sulphate (+Cu or −Cu) to modulate expression of transfected *Dfz*2 from the metallothionein promoter[45]. Cellular proteins were analysed on blots with antibodies against Arm (upper panel). Incubation with Wg-containing medium produces an increase in the level of Arm in clone 8 cells and in *Dfz*2-expressing S2 cells, but not in untransfected S2 cells. Further induction of *Dfz*2 expression in transfected cells by preincubation with copper sulphate leads to a lower response to Wg. As a control for loading, blots were stripped and incubated with antiserum against α-catenin (lower panel).
METHODS. To produce control conditioned medium or conditioned medium containing soluble Wg protein, untransfected S2 cells or S2 cells stably transfected with a construct in which the *wg* coding region is under the control of a heat-shock promoter were used as described[16]. S2 cells stably transfected with *Dfz*2 under the control of the metallothionein promoter were generated by hygromycin selection following transfection with a plasmid carrying the *Dfz*2 coding region inserted into pMK33[45]. Clone 8, S2, and *Dfz*2 transfected S2 cells were incubated with concentrated medium for 2 h. After the incubation, cells were lysed and protein extracts analysed using a monoclonal anti-armadillo antibody 7A1 (ref. 7) or rat-monoclonal anti-α-catenin antibody DCAT-1 (ref. 46). Bound antibody was visualized using the ECL system (Amersham).

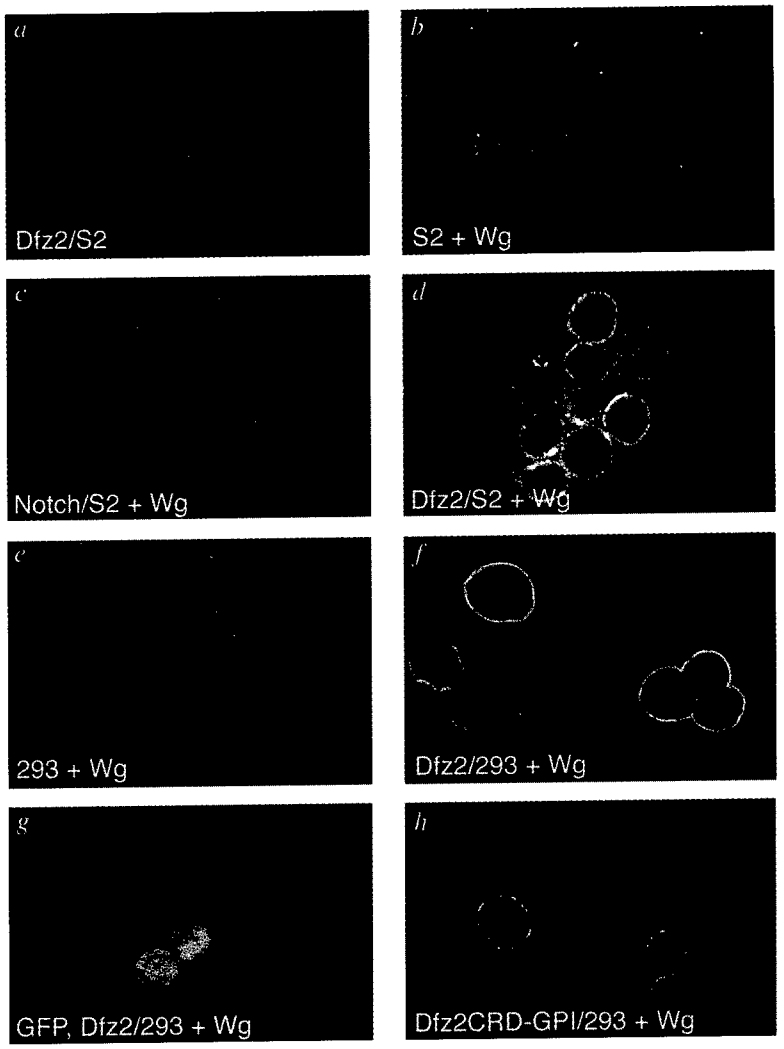


FIG. 4 Wg protein binds to cells transfected with *Dfz*2. Untransfected and transfected cells were incubated with concentrated conditioned medium from untransfected S2 cells (*a*) or from S2 cells producing Wg (*b–h*; see Fig. 3 legend). Following incubation with conditioned medium, the cells were washed, fixed in paraformaldehyde/PBS, and incubated with an anti-Wg antibody directed against an 85-amino-acid domain that is found in Wg but absent from all other Wnt proteins[4]. This domain is dispensable for Wg activity (Chi-hwa Wu, C.H.S. and R.N., unpublished observations). Untransfected S2 cells (*b*), S2 cells transfected with a *Notch* expression plasmid (c), and untransfected 293T cells (human embryonic kidney-cell line 293 stably expressing SV40 TAg; *e*) show a low level of fluorescent antibody binding. *d*, Roughly 80% of S2 cells stably transfected with *Dfz*2 and incubated with Wg show anti-Wg antibody binding to the cell surface. *f*, 10–20% of 293T cells transiently transfected with a *Dfz*2 expression plasmid and incubated with Wg show anti-Wg antibody binding to the cell surface. *g*, 293T cells cotransfected with a mixture of Dfz2 expression plasmid and a GFP expression plasmid, and incubated with Wg and anti-Wg antibodies show colocalization of green cytosolic fluorescence (GFP) and red surface fluorescence (Wg and anti-Wg antibody) and confirm that 10–20% of cells were transfected, and that only this subset of cells bind Wg. *h*, 293 cells contransfected with a T-antigen expression plasmid and a truncated Dfz2 construct in which the signal sequence, the CRD, and the first half of the linker region are anchored to the cell surface by GPI, show cell-surface binding to Wg and anti-Wg antibody.
METHODS. Untransfected S2 cells and S2 cells expressing *Dfz*2 were washed twice in PBS and incubated with 1.5 ml of 10× concentrated conditioned medium at 4 °C for 3 h. After three 10-min washes with cold PBS the cells were fixed in 2% paraformaldehyde (Polysciences, Inc.) for 15 min at room temperature. After three more 10-min washes with PBS, affinity purified anti-Wg antibody diluted 1:25 in 5% donkey serum/PBS was added to the cells and incubated overnight at 4 °C. After additional washes in PBS, the cells were incubated with fluorescent Cy3 secondary antibody (Jackson Immunoresearch) and mounted. For transient expression in 293T or 293 cells, the *Dfz*2 coding region was inserted into the pCIS expression vector under the control of the cytomegalovirus immediate early promoter/enhancer and with an optimized translation-initiation context, and transfected into 293T or into 293 cells with a T-antigen expression plasmid using the calcium phosphate method[47]. Eight hours after transfection, 10 mM chlorate was added. Twenty-four hours later, the cells were treated with 20 mU of heparatinase (Seikagaku) for 3 h before adding Wg protein. S2 cells expressing Notch[48] were obtained from S. Artavanis-Tsakonas. From N to C-termini, the GPI-anchored construct consists of the first 270 amino acids of Dfz2, a myc epitope[49] and the C-terminal 40 amino acids of decay activating factor, a GPI-anchored protein[50]. Confocal images were collected with a Bio-Rad MRC 1000 confocal laser attached to a Zeiss Axio scope microscope. The same number of scans (20) were taken to visualize the fluorescence of each sample. Images were processed in Adobe Photoshop 3.0.

Wg and anti-Wg antibodies (Fig. 4*d*), whereas *Notch* transfected or non-transfected cells incubated either with or without Wg show a background of randomly distributed spots of low fluorescence intensity (Fig. 4*b*, *c*). *Dfz*2-transfected cells incubated in the absence of Wg show a similar low-intensity, spotty background (Fig. 4*a*). We conclude that Wg specifically binds to S2 cells expressing *Dfz*2.

Although this binding experiment indicates that Wg and Dfz2 probably interact directly, it is possible that expression of *Dfz*2 could act indirectly by inducing or unmasking a Wg receptor. We therefore performed a series of binding experiments using heterologous cells, in this case human embryonic kidney cells (293 or 293T; both will be referred to as 293) and a variety of wild-type and mutant fz constructs. Pretreatment of the 293 cells with chlorate and heparatinase[27] lowered the overall background of Wg binding (presumably binding of Wg to extracellular matrix molecules; Fig. 4*e*), and revealed specific binding of Wg to the surface of 293 cells that had been transiently transfected with *Dfz*2 (Fig. 4*f*) but not to untransfected cells or cells that had been transfected with a bovine rhodopsin expression construct (Fig. 4*e*, and data not shown). In a second experiment in which 293 cells were cotransfected with a green fluorescent protein (GFP) expression plasmid and the *Dfz*2 expression plasmid, we observed that cells with green cytosolic fluorescence (caused by GFP) also had red surface fluorescence (Wg and anti-Wg antibody; Fig. 4*g*).

Each frizzled protein has an extracellular cysteine-rich domain (CRD) that is joined to the transmembrane domain by a variable linker. The CRD has been proposed to constitute part or all of the ligand-binding domain[18], which suggests that cell-surface expression of the isolated CRD segment might confer Wg binding. This possibility was tested by expressing a truncated form of Dfz2 in which the CRD and part of the linker region was displayed on the cell surface as a glycosylphosphotidylinositol (GPI)-anchored protein. This protein was detected at the surface of transfected cells by immunostaining either with antibodies directed against the Dfz2 extracellular domain or with antibodies to a myc epitope tag that was engineered near the C terminus of the GPI-anchored protein (data not shown). When 293 cells transfected with the GPI-anchored Dfz2 CRD were incubated with Wg and anti-Wg antibodies, strong surface staining was observed (Fig. 4*h*). We conclude from this experiment that the Dfz2 CRD constitutes either all, or a significant part of, the ligand-binding domain.

Transfection of a subset of *frizzled* members

In many ligand–receptor systems a single ligand can bind to more than one species of receptor, or a single receptor can bind to more than one species of ligand, or both. Among G-protein-coupled receptors there are many examples of receptor subtypes that recognize the same ligand but differ in effector coupling, tissue distribution and pharmacology. In the fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β/activin/inhibin systems, tissue-culture experiments show that different receptors can bind to a single ligand and that different ligands can bind to a single receptor[28–30].

As a first step in examining the question of ligand–receptor specificity in the Wnt–frizzled system, we tested the ability of Wg to bind to 293 cells transfected with *Drosophila fz* and with six mammalian *frizzled* sequences[18]. 293 cells transfected either with *fz*, human *fz*5 (*Hfz*5), or mouse *fz*4, *fz*7 or *fz*8 (*Mfz*4, *Mfz*7 and *Mfz*8) bind added Wg (Fig. 5*a*), whereas transfection with *Mfz*3 and *Mfz*6 did not confer Wg binding (Fig. 5*b*, *c*). As a complement to the Dfz2 CRD GPI-anchor experiment, a derivative of *Mfz*4 was constructed in which the CRD was replaced with a myc epitope. In transfected 293 cells, immunostaining with an anti-myc antibody, and western blotting with an antibody specific for the *Mfz*4 C terminus show, respectively, that the CRD-deleted Mfz4 protein accumulated at the cell surface and to the same percentage of membrane protein as full-length Mfz4 (Fig. 5*e* and data not shown). However, CRD-deleted Mfz4 did not confer Wg binding (Fig. 5*d*). This experiment further implicates the CRD as an essential determinant of Wg binding. Figure 6 summarizes all of the Wg–frizzled binding experiments described above.

Discussion

The experiments reported here identify a second member of the *frizzled* family in *Drosophila*, *Dfz*2, and show by the following two criteria that it can function as a receptor for Wg. First, transfection of S2 cells with *Dfz*2 confers Wg responsiveness as determined by an increase in cytoplasmic Arm concentration, and second, transfection with *Dfz*2 confers cell-surface binding of Wg in both homologous (S2) and heterologous (293) cell systems. It is important to note that these data do not rule out the possibility that additional molecules in the conditioned medium might associate with Wg and participate in its binding to the receptor. They also do not rule out the possibility that Dfz2 is part of a larger complex at the cell surface; in such a complex Dfz2 would be necessary but may not be sufficient for binding and/or signal transduction. We note that these experiments do not support the proposal that Notch is the Wg receptor[31], because *Notch*-

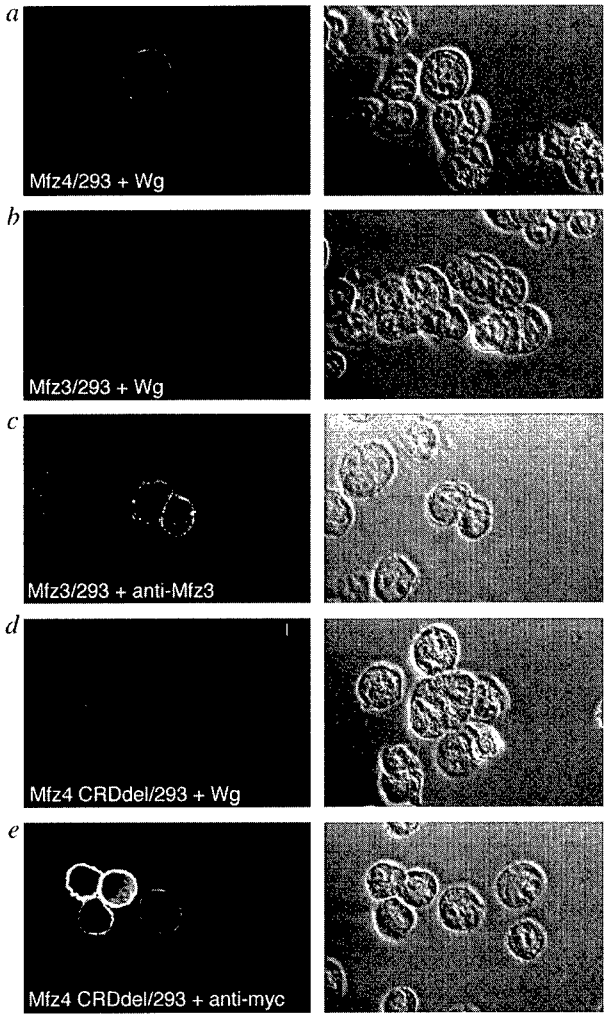
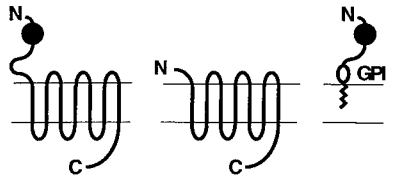


FIG. 5 Binding of Wg to 293 cells transfected with mammalian *frizzled* family members. 293 cells were cotransfected with a T-antigen expression plasmid and the following coding segments inserted into the pCIS vector: *a*, Mfz4; *b*, *c*, Mfz3; *d*, *e*, Mfz4 with the CRD-replaced by a myc epitope. Cells were incubated with Wg and anti-Wg antibodies (*a*, *b*, *d*), affinity-purified antibodies directed against the extracellular domain of Mfz3 (amino acids 1–205; c), or anti-myc antibodies (e). In each pair of photographs the left hand panel shows the immunostaining and the right hand panel the corresponding phase-contrast image.
METHODS. As described in Fig. 4.



fz	+		
Dfz2	+		+
Mfz3	-		
Mfz4	+	-	
Hfz5	+		
Mfz6	-		
Mfz7	+		
Mfz8	+		

FIG. 6 Summary of Wg–frizzled interactions. Left, intact frizzled protein; centre, frizzled protein with the CRD deleted; right, the frizzled CRD and part of the linker region anchored to the membrane by GPI. + and – indicate the presence or absence of cell-surface binding by Wg after transfection of the frizzled proteins listed on the left. The filled ball represents the CRD. D, *Drosophila*; M, mouse, H, human. Whether Mfz6 is produced and transported to the cell surface has not been determined.

transfected cells do not bind Wg (Fig. 4*c*), nor does Notch confer a Wg-dependent increase in Arm (F. van Leeuwen and R.N., data not shown).

At present, there is no *in vivo* evidence that *Dfz2* is required for Wg signalling, as there are no known *Dfz2* mutants. Although the pattern of *Dfz2* expression is suggestive of its participation in *wg* signalling at multiple points in development, definitive evidence of that participation will require a genetic analysis of *Dfz2* function. The degree to which the *Dfz2* mutant phenotype resembles the *wg* phenotype will most probably depend on whether additional Wg receptors exist *in vivo*.

The ability of Dfz2 to function as a Wg receptor implies more generally that other members of the *Wnt* and *frizzled* families are linked in receptor–ligand relationships. The observation that *Drosophila fz* and some members of the mammalian *frizzled* family also confer Wg binding supports this inference but also suggests that there may be overlapping specificities in Wnt–Frizzled interactions. From the general conclusion that *frizzled* family members encode Wnt receptors, we infer that *in vivo* the *Drosophila fz* protein recognizes at least one Wnt other than Wg (three of which are known[32–34]), and, by extension, that the initial biochemical steps in *fz*-mediated tissue polarity signalling resemble the initial steps of *wg*-mediated segment polarity signalling.

The experiments reported here provide a new point of entry for examining the biochemistry of Wnt signalling. It should now be possible to determine which cytoplasmic proteins interact directly with the frizzled receptors, whether these interactions are modified by Wnt binding, and whether Wnt signalling is regulated by covalent or non-covalent receptor modification. It is interesting to note that many Fz proteins, including Dfz2, contain a S/T-X-V motif at their C-terminal end; this motif has been shown to interact with PDZ (or DHR) domains in a variety of proteins[35]. Dsh, one of the cytoplasmic components of Wg signalling, contains a PDZ domain[9,10].

A question remaining is how frizzled and Wnt proteins might interact to initiate signal transduction. One attractive hypothesis is suggested by the relative immobility of Wnt proteins because of their affinity for the extracellular matrix, and the predicted mobility of the CRD, which we show here constitutes part or all of the ligand-binding site. The prediction that the CRD is mobile follows from the predicted lack of a stable structure in the highly divergent sequence that links it to the membrane-embedded domain. For example, in Dfz2 this linker region includes a stretch of 42 amino acids that includes 21 glycines and 15 serines. Therefore the CRD may be able to bind to an extracellular matrix-associated Wnt protein at a distance of several tens of nanometres from the plasma membrane of the cell on which the frizzled receptor resides. It is tempting to speculate that binding of a Wnt ligand to the CRD disrupts or modifies an interaction between the CRD and the extracellular face of the transmembrane domain, and that this results in a rearrangement of transmembrane α-helices. Although the frizzled proteins have no primary sequence homology to G-protein-coupled receptors[18], this allosteric model suggests a mode of receptor activation that is reminiscent of that proposed for G-protein-coupled receptors[36]. □

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CORRESPONDENCE and requests for materials should be addressed to J.N. or R.N. (e-mail: jeremy.nathans@qmail.bs.jhu.edu or rnusse@cmcm.stanford.edu).

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wingless signaling in the *Drosophila* eye and embryonic epidermis

Kenneth M. Cadigan and Roel Nusse

Howard Hughes Medical Institute and the Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305, USA

SUMMARY

After the onset of pupation, sensory organ precursors, the progenitors of the interommatidial bristles, are selected in the developing *Drosophila* eye. We have found that *wingless*, when expressed ectopically in the eye via the *sevenless* promoter, blocks this process. Transgenic eyes have reduced expression of *acheate*, suggesting that *wingless* acts at the level of the proneural genes to block bristle development. This is in contrast to the wing, where *wingless* positively regulates *acheate* to promote bristle formation. The *sevenless* promoter is not active in the acheate-positive cells, indicating that the *wingless* is acting in a paracrine manner. Clonal analysis revealed a requirement for the genes *porcupine*, *dishevelled* and *armadillo* in mediating the *wingless* effect. Overexpression of *zeste white-3* partially blocks the ability of *wingless* to inhibit bristle formation, consistent with the notion that *wingless* acts in opposition to *zeste white-3*. Thus the *wingless* signaling pathway in the eye appears to be very similar to that described in the embryo and wing. The *Notch* gene product has also been suggested to play a role in *wingless* signaling (J. P. Couso and A. M. Martinez Arias (1994) *Cell* 79, 259-72). Because *Notch* has many functions during eye development, including its role in inhibiting bristle formation through the neurogenic pathway, it is difficult to assess the relationship of *Notch* to *wingless* in the eye. However, we present evidence that *wingless* signaling still occurs normally in the complete absence of Notch protein in the embryonic epidermis. Thus, in the simplest model for *wingless* signalling, a direct role for *Notch* is unlikely.

Key words: *wingless*, signal transduction, *Notch*, *Drosophila*, neurogenesis, segment polarity

INTRODUCTION

The *wingless* (*wg*) gene is the best characterized member of the *Wnt* family, which contains over fifty genes in organisms ranging from nematodes to humans (Nusse and Varmus, 1992). *Wnt* genes encode cysteine-rich proteins containing signal sequences and several members, including wg, have rigorously been shown to be secreted (Bradley and Brown, 1990; Fradkin et al., 1995; González et al., 1991; Papkoff and Schryver, 1990; Van den Heuvel et al., 1989; Van Leeuwen et al., 1994).

In *Drosophila melanogaster*, *wg* is required throughout embryogenesis and larval development for a wide range of patterning events (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Some of these include specifying cell fate in the embryonic epidermis (Baker, 1988; Bejsovec and Martinez-Arias, 1991; Dougan and Dinardo, 1992), CNS (Chu-Lagraff and Doe, 1993), mesoderm (Baylies et al., 1995; Wu et al., 1995) and endoderm (Hoppler and Bienz, 1995). In larval development, *wg* is required for patterning in leg (Couso et al., 1993; Diaz-Benjumea and Cohen, 1994; Struhl and Basler, 1993; Wilder and Perrimon, 1995) and wing (Couso et al., 1994; Diaz-Benjumea and Cohen, 1995; Phillips and Whittle, 1993) imaginal discs. In the eye, *wg* has recently been shown to be necessary for proper spacing of morphogenetic furrow initiation (Ma and Moses, 1995; Treisman and Rubin, 1995). How one signal can produce so many responses remains an important unanswered question in developmental biology.

Consistent with being a secreted molecule, wg is thought to execute most of its functions in a paracrine manner. In the best documented cases, the range of wg action can vary from one (Vincent and Lawrence, 1994) to several (Hoppler and Bienz, 1995) cell diameters, though the exact limits of wg diffusion remain unclear (Axelrod et al., 1996; Peifer et al., 1991; Theisen et al., 1994). In a few cases, wg regulates gene expression in the same cells in which it is expressed, e.g. the activation of *cut* expression at the wing margin (Couso et al., 1994) and the regulation of its own expression in the embryo (Bejsovec and Wieschaus, 1993; Hooper, 1994; Yoffe et al., 1995). This embryonic autoregulation has been referred to as 'autocrine wg signaling' but it is not clear whether wg works in a truly autocrine manner. However, recent evidence indicates that *wg* autoregulation may have different genetic requirements than the paracrine signaling pathway of wg (Hooper, 1994; Manoukian et al., 1995; see discussion).

Three genes with embryonic phenotypes very similar to that of *wg* have been described (Klingensmith et al., 1989; Peifer and Wieschaus, 1990; Perrimon et al., 1989; Perrimon and Mahowald, 1987), *porcupine* (*porc*), *dishevelled* (*dsh*) and *armadillo* (*arm*). Another gene, *zeste white-3* (*zw3*; also known as *shaggy*) has a mutant phenotype (Perrimon and Smouse, 1989; Siegfried et al., 1992) very similar to that of embryos

where *wg* has been expressed ubiquitously (Noordermeer et al., 1992). Genetic epistasis (Noordermeer et al., 1994; Peifer et al., 1994b; Siegfried et al., 1994) have ordered these genes in the following genetic pathway:

porc → *wg* → *dsh* —| *zw3* —| *arm*

porc has been shown to be involved in either secretion or subsequent diffusion of the wg protein (Siegfried et al., 1994; van den Heuvel et al., 1993a) and the other three genes are thought to be required for receiving the *wg* signal (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994).

Recent work has revealed that many aspects of this embryonic *wg* signaling pathway are conserved in larval *Drosophila* tissues as well as in other organisms. Analysis of *dsh*, *zw3* and *arm* mutations in leg and wing imaginal discs indicates that these genes are required for *wg* signaling (Couso et al., 1994; Diaz-Benjumea and Cohen, 1994; Klingensmith et al., 1994; Peifer et al., 1991; Theisen et al., 1994). This has been best shown in the developing wing margin, where these genes mediate *wg* regulation of the *acheate* (*ac*) gene (Couso et al., 1994; Blair, 1994). The vertebrate homologs of these three genes have been shown to play a role in inducing dorsal mesoderm in *Xenopus* in a manner consistent with functioning in a *Wnt* signaling pathway (Dominguez et al., 1995; He et al., 1995; Heasman et al., 1994; Pierce and Kimelman, 1995; Rothbacher et al., 1995; Sokol et al., 1995).

The *wg* signaling pathway described above was first postulated based on extensive genetic analysis, but recent work indicates that some of the gene products may function directly with wg in a biochemical pathway. The *arm* gene encodes the *Drosophila* homolog of β-catenin (Peifer and Wieschaus, 1990), a component of vertebrate adherens junctions (Kemler, 1993). A similar junctional complex is found in flies (Peifer, 1993) but a substantial pool of cytoplasmic arm protein also exists (Peifer et al., 1994b; Van Leeuwen et al., 1994). *wg* signaling causes an accumulation of cytoplasmic arm protein (Peifer et al., 1994b; Van Leeuwen et al., 1994) caused by a dramatic decrease in arm protein turnover (Van Leeuwen et al., 1994). This accumulation is correlated with a reduction in phosphorylation of arm (Peifer et al., 1994a). This increase in arm protein is thought to somehow transduce the *wg* signal to the nucleus (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994).

Consistent with the proposed genetic pathway, mutations in the other components of the *wg* pathway affect arm protein levels. The normal segmentally repeated accumulation of arm protein is absent in *wg*, *porc* and *dsh* mutants (Peifer et al., 1994b; Riggleman et al., 1990), while *zw3* mutants have uniformly high levels of arm protein (Peifer et al., 1994b; Siegfried et al., 1994). The *dsh* gene encodes a novel protein (Klingensmith et al., 1994; Theisen et al., 1994) containing a PDZ domain (Kennedy, 1995) that is phosphorylated in response to *wg* in embryos and cultured cells, and this phosphorylation is correlated with the ability of *dsh* to stabilize the arm protein (Yanagawa et al., 1995). *zw3* encodes a serine-threonine protein kinase that is homologous with mammalian glycogen synthase kinase-3 (Ruel et al., 1993a; Siegfried et al., 1992). At the present time, it is not clear whether any of the regulatory steps in the pathway are direct or how many missing components remain to be identified.

One new candidate for functioning in the *wg* pathway is the product of the *Notch* (*N*) gene, which encodes a transmembrane protein found on the surface of cells. N protein is thought to act as the receptor for the *Delta* (*Dl*) gene product in a signaling pathway involved in many aspects of development (Muskavitch, 1994; Artavanis-Tsakonas et al. 1995). Its potential role in the *wg* pathway is based on strong genetic interactions between *N* and *wg* mutations in several tissues, but primarily in the wing (Couso and Martinez Arias, 1994; Hing et al., 1994). It is possible that the role of *N* in the separate but oft-used pathway with *Dl* could mask a requirement for *N* in *wg* signaling when *N* mutant embryos or clones are examined. Because N is expressed at the cell surface and appears to act as a receptor, it has been postulated that *wg* encodes a ligand for the N protein (Couso and Martinez Arias, 1994).

This report describes a phenotype created by ectopic expression of *wg* during eye development. These transgenic animals lack the mechanosensory bristles normally surrounding each facet of the compound eye. This is the exact opposite effect seen in the wing, where *wg* is required for bristle formation (Couso et al., 1994; Phillips and Whittle, 1993). Despite this difference in regulation, the *wg* signal transduction machinery found in the embryo and wing also functions in the eye. Finally, the role of *N* in *wg* signaling was examined in the eye and in the embryonic epidermis, where, in the complete absence of N protein, *wg* signaling appears to occur normally. These data argue against a direct role for *N* in *wg* signaling.

MATERIALS AND METHODS

Fly stocks

The mutant alleles in components of the *wg* signaling pathway used in this study were: wg^{IL}, wg^{IN}, wg^{CX4}, $porc^{I8}$, $porc^{2E}$, dsh^{V26}, dsh^{477}, arm^{XM19}, arm^{25B}, $sgg^{D127}$and $zw3^{M11}$. wg^{CX4} (van den Heuvel et al., 1993a,b), dsh^{V26} (Yanagawa et al., 1995) and sgg^{D127} (Ruel et al., 1993b) are null alleles, wg^{IN} encodes a non-secreted wg protein (van den Heuvel et al., 1993a,b), $wg^{IL}$is a temperature-sensitive allele (Baker, 1988) and the rest are characterized phenotypically as strong alleles (Klingensmith, 1993; Siegfried et al., 1992), except for the *arm* alleles, which are hypomorphs but are the strongest alleles that are cell viable when homozygous (Peifer et al., 1991). Two null alleles of *N*, $N^{264.40}$ and N^{5419} (S. Artavanis-Tsakonas, personal comm.) and the temperature-sensitive alleles N^{ts1} (Cagan and Ready, 1989b), Dl^{6E} (Dietrich and Campos-Ortega, 1984) and Dl^{RF} (Parody and Muskavitch, 1993) were also used. For further information, see Lindsley and Zimm (1992).

A P-element construct placing the *wg* ORF under the control of the *sevenless* (*sev*) promoter (P[*sev-wg*]) was made by inserting the *XbaI*/*Cla*I (blunt ended) fragment of the *wg* cDNA, pCV (Rijsewijk et al., 1987) into the *Xba*I and *Bgl*II (blunt ended) sites of pSEWa (Fortini et al., 1992), between the *sev* proximal promoter and 3′ processing elements. pSEWa also contains three tandem repeats of the *sev* enhancer 5′ of the promoter. yw^{67} embryos were coinjected with P[*sev-wg*] and pπ25.7 as described previously (Rubin and Spradling, 1982) and several independent lines were established using standard balancer stocks. A stock containing the *lacZ* coding sequences under the control of the *sev* enhancer (three tandem repeats) and *hsp70* proximal promoter (P[*sev-lacZ*]; R. Carthew, personal communication) was obtained from Todd Laverty (UC Berkeley, CA).

The following heat-shock strains were used: P[*hs-wg*] (Noordermeer et al., 1992), P[*hs-zw3*] (Siegfried et al., 1992) and P[*hs-dsh*] (Axelrod et al., 1996). P[*hs-wg*] is on the third chromosome, the other two on the second. The following chromosomes were created by recombination. P[*sev-wg*; w^-], P[*hs-zw3*; w^+] (the *white* (*w*) gene in

the P[*sev-wg*; w^+] transgene was inactivated by EMS mutagenesis). A P[*sev-wg*] insert on chromosome 3L was recombined with a Dl^{RF} mutation to make P[*sev-wg*], Dl^{RF}. Two different P[*hs-dsh*; w^+], wg^{IL} recombinants were created, one using a wg^{IL} *cn bw sp* chromosome and the other a wg^{IL} *br pr*, since both chromosomes contain a different lethal mutation unrelated to *wg* (Couso et al., 1994). Both P[*hs-dsh*], wg^{IL} recombinants were placed over a SM5a-TM6B compound chromosome, so that homozygotes could be identified by the absence of the *Tubby* pupal marker.

Whole-mount stainings of pupal eyes and embryos

Pupal eyes were dissected and then immunostained as described (Blochlinger et al., 1993). Embryo stainings were performed essentially as previously described [Frasch et al., 1987; Grossniklaus et al., 1992). Affinity-purified rat α-*cut* antisera was generously provided by K. Blochinger (Fred Hutchinson Institute, WA), mouse α-*ac* monoclonal antibody was a gift of Sean Carroll (University of Wisconsin at Madison). Rabbit α-*lacZ* antisera was from Cappel and affinity purified rabbit α-*wg* antisera was kindly provided by C. Harryman-Samos (Stanford University, CA). Mouse α-*N* monoclonal antibody was provided by S. Artavanis-Tsakonas (Yale Univ. CT) and mouse α-*en* antisera by T. Kornberg (UCSF, CA). The primary antibodies were used at the following dilutions: *ac*, 1:3 to 1:5, *wg*, 1:20, *N*, 1:100, *cut* and *en*, 1:300, *lacZ*, 1:500. For histochemistry, secondary antibodies were either biotinylated (goat α-mouse, horse α-rabbit and rabbit α-rat; all from the Elite ABC kit, Vectastain, used at a 1:500 dilution) or goat α-rabbit conjugated to alkaline phosphatase (from Vector, used at 1:300). For fluorescence microscopy either donkey FITC α-mouse (1:100) or donkey Cy3 α-rabbit (1:200) were used (Jackson Immunochemicals). Confocal images were collected with a Bio-Rad MRC 1000 confocal laser setup attached to a Zeiss Axioscope microscope. Images were imported into Adobe Photoshop for presentation.

In situ hybridization to whole-mount embryos using digoxigenin-labeled probes (Tautz and Pfeiffle, 1989) and antibody/in situ double stainings (Manoukian and Krause, 1992) were performed as described (detailed protocol available upon request).

All whole-mount stainings were photographed with a Nikon Microphot-FXA microscope and slides were scanned into Adobe Photoshop for presentation.

Production of mosaic animals

Mutant alleles of *dsh*, *zw3* and *arm* were recombined unto a P[*hs-neo*; *FRT*]18A chromosome, *porc* onto P[*hs-neo*; *FRT*]19A, *wg* onto P[*hs-neo*; *FRT*]40A and a P[*sev-wg*; w^+] mapping to 3L onto P[*hs-neo*; *FRT*]80A, all in a *w* background. *w* clones were induced in animals heterozygous with the appropriate P[*mini-w*$^+$], P[*FRT*] chromosome: P[*mini-w*$^+$; *hs-πM*]5A, 10D, P[*hs-neo*; *FRT*]18A; P[*mini-w*$^+$]18A, P[*hs-neo*; *FRT*]19A; P[*mini-w*$^+$; *hs-πM*]21C, 36F, P[*hs-neo*; *FRT*]. All FRT derivatives are as described (Xu and Rubin, 1993) except for P[*mini-w*$^+$]18A, which is from the Jan lab enhancer detection collection (Bier et al. 1989). FLP recombinase was provided from the FLP-99 chromosome (Chou and Perrimon, 1992). Clones were induced by a one hour heat shock (37°C) 24-48 hours (at 25°C) after egg laying and scored for the absence of pigmentation in the adult eye.

For production of *N* germ-line clones, the *N* null alleles were recombined onto a P[*mini-w*$^+$; *FRT*]^{101} chromosome (Chou and Perrimon, 1992). *N*, P[*mini-w*$^+$; *FRT*]^{101}/FM7 females were crossed to a w ovo^{D1}, P[*mini-w*$^+$; *FRT*]^{101}/Y ; P[*hs-FLP*]^{38} stock (Chou and Perrimon, 1992) and progeny were heat shocked late 3rd instar/early pupation for 2 hours at 37°C (earlier heat shocks resulted in high lethality due to somatic clones). Mosaic mothers were crossed to P[*ftz-lacZ*]C males (Hiromi and Gehring, 1987) or P[*ftz-lacZ*]C; P[*hs-wg*]/TM3 males. Embryos with no β-gal staining lacked both maternal and zygotic expression of *N*.

Heat shocks and other temperature shifts

The P[*hs-wg*] phenotype was induced by multiple heat shocks as previously described (Noordermeer et al., 1992). Late larval/early pupal temperature shifts were performed by submerging glass vials in a water bath of the appropriate temperature (37°C for heat shocks). At all other times, larvae and pupae were kept at 25°C. Formation of white pupae was used as the reference point (0 hours APF).

Histology

Flies were prepared for scanning electron microscopy by serial dehydration in ethanol and Freon 113 (EM Sciences) as described (Kimmel et al., 1990). Dried samples were mounted with colloidal graphite, and a 10 nm gold-platinum coat was applied with a Hummer sputter coater. The samples were viewed with an AMR1000 SEM and photographed using Polapan 400 film (Kodak). Pupal eyes were surface stained with $Co(NO_3)_2{\cdot}6H20$ and $(NH_4)_2S$ as described (Kimmel et al., 1990).

RESULTS

wg blocks SOP formation in the eye

During the course of our attempts to create a dominant adult *wg* mutant through limited misexpression of *wg* during larval development, we found a highly penetrant phenotype when *wg* was placed under the control of the eye-specific promoter *sev*. As shown in Fig. 1, the eyes of P[*sev-wg*] flies appear normal, except that the interommatidial bristles, normally found at alternating vertices in the compound eye's hexagonal array, are almost completely missing. Sections through adult eyes (data not shown) and surface staining of pupal eyes with cobalt sulfide (Fig. 1E,F) revealed no other detectable abnormality in

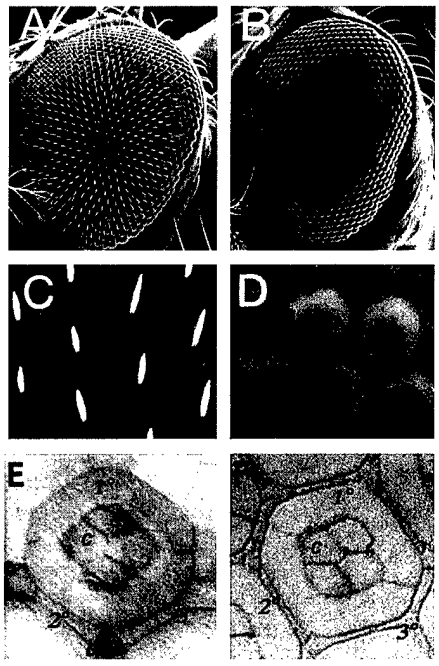

Fig. 1. P[*sev-wg*] flies lack interommatidial bristles. SEM images of parental yw^{67} (A,C) or P[*sev-wg*] (B,D) eyes showing lack of both the base and shaft of the bristles. Note that the hexagonal array and surface of each facet are unaffected in the transgenic eyes. Cobalt sulfide staining of pupal eyes (36 hours APF at 25°C) of control (E) and P[*sev-wg*] (F). Cone cells (c) and the 1°, 2° and 3° pigment cells appear normal in transgenic eyes, but a 3° pigment cell is found in place of each bristle (b).

adult eyes. The bristles are replaced in the repeated structure of the eye with tertiary pigment cells. Thus, at the level of ectopic *wg* expressed from the P[*sev-wg*] transgene, the effect of *wg* on eye development is very specific.

Interommatidial bristles are mechanosensory organs composed of four cells that are derived from a single sensory organ precursor (SOP; (Cagan and Ready, 1989a). Larval SOP determination has been best described in the wing imaginal disc (Campuzano and Modolell, 1992; Jan and Jan, 1993b). The process begins with small groups of cells expressing basic helix-loop-helix proteins such as *acheate* (*ac*) and *scute* (Cubas et al., 1991; Skeath and Carroll, 1991). All the cells in these proneural clusters have the ability to become the SOP, however, in a wild-type background, only one does. This cell is thought to become the SOP by reaching a threshold level of *ac* and/or *scute* after which it inhibits these genes' expression in its neighbors (Ghysen et al., 1993; Simpson, 1990). This lateral inhibition is mediated by the neurogenic pathway, in which the products of the *Dl* and *N* genes are thought to act as ligand and receptor, respectively (Artavanis-Tsakonas et al., 1995; Muskavitch, 1994). The initiation of SOP development is correlated with the expression of a new set of genes, such as *neuralized* (Huang and Dambly-Chaudière, 1991) and for some SOPs, *cut* (Blochlinger et al., 1993). The SOP undergoes to two divisions to generate the four cells that will give rise to the mature bristle organ (Bodmer et al., 1989; Hartenstein and Posakony, 1989).

The events leading to SOP formation in the eye have many similarities to those occuring in other tissues. ac protein becomes detectable shortly after white prepupa formation (data not shown). At 3 hours after the white prepupa stage (3 hours APF), the *ac* gene is expressed in small clusters of cells throughout the eye (Fig. 2C). Unlike the photoreceptors and cone cells, the appearance of the ac-positive cells is not related to the distance from the morphogenetic furrow, although the cells anterior of the furrow do not express *ac* (see arrows in Fig. 2C). By 6 hours APF, only one cell per cluster still expresses *ac*, again with the anterior-most portion of the eye showing a less mature pattern (data not shown). At 15 hours APF, after the eye disc everts, ac protein is gone, but the daughters of the SOPs can be observed by staining with α-*cut* antisera (Fig. 2A). Because of the complicated morphogenetic movements associated with the eye/head disc eversion, we have been unable to stain tissue between 6 and 15 hours APF.

In the P[SEV-wg] eyes, *ac* expression is greatly reduced compared to controls though not completely absent (Fig. 2D,F). After disc eversion, no SOPs are found, as judged by *cut* staining (Fig. 2B) and an enhancer detector line for the *neuralized* gene (data not shown). Thus, *wg* appears to act at the level of the proneural genes, i.e., *ac*, to inhibit SOP formation.

wg-dependent SOP inhibition is a paracrine effect

The activity of the *sev* promoter has been well studied in third instar larva, by monitoring endogenous sev expression (Tomlinson et al., 1987) and with chimeric constructs (Bowtell et al., 1989) using *sev* enhancer and promoter elements similar to the ones in P[*sev-wg*]. The enhancer is active in the cone cells and in a subset of the underlying photoreceptor precursors. No description of *sev* expression has been reported after pupation, so the possibility existed that *wg* was expressed in the proneural cells of P[*sev-wg*] eyes, suggesting a possible autocrine effect.

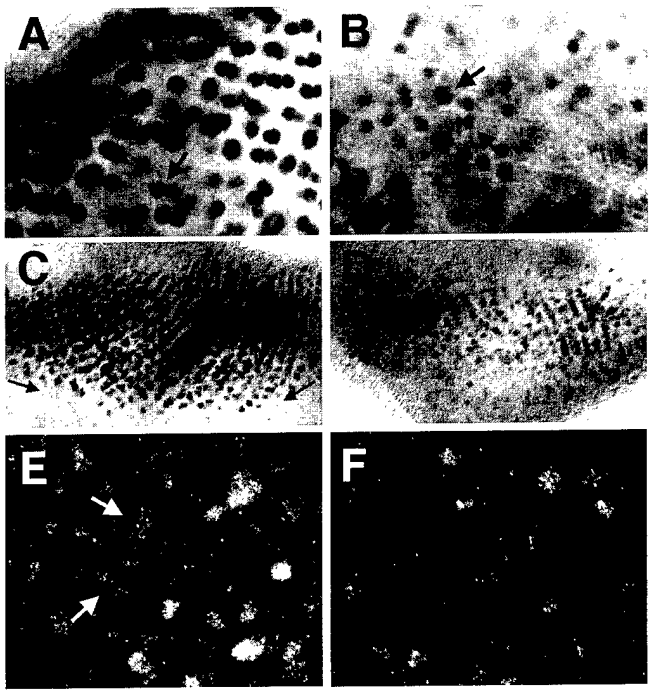

Fig. 2. P[*sev-wg*] eyes have lower than normal levels of ac protein and no SOPs. yw^{67} (A,C,E) and P[*sev-wg*] (B,D,F) pupal eyes were stained with antibodies against cut (A,B; 15 hours APF) or ac (C-F; 3 hours APF). E and F are confocal images. Pairs of cut-positive SOP daughter cells (see arrows) can be seen in controls (A) but not in the transgenic eyes (the arrows point to cone cells, which also express *cut* and lie in a slightly more apical focal plane). *ac* is expressed basally in small clusters up until the morphogenetic furrow (C; arrows indicate the approximate position of the furrow. Anterior is down). The ac-positive clusters usually consist of two or three cells (E; see arrows). ac protein remains in P[*sev-wg*] eyes to varying degrees (the image in D lies in the middle of the range; F shows a close up of an area with relatively high levels of *ac* expression), but staining is always significantly less than controls (E).

This question was addressed by examining the distribution of wg protein in P[*sev-wg*] eyes. Though wg is a secreted protein, it is found at the highest levels on the surface of the same cells that synthesize it (Bejsovec and Wieschaus, 1995; Couso et al., 1994; van den Heuvel et al., 1993). In P[*sev-wg*] eyes, the highest levels of wg protein were found around the four cone cells (Fig. 3A) and accumulated on their apical surface (Fig. 3B). In more basal sections of the eye, wg protein was associated with the photoreceptors, which extend basally to the same plane as the ac-positive cells (Fig. 3C). There was no significant overlap between wg protein and the remaining cells expressing *ac*.

To confirm that the sev enhancer was not active in the proneural clusters, we stained eyes of flies that contained a P[*sev-lacZ*] transgene (see Materials and Methods) for products of *lacZ* and *ac*. As found for *wg* in P[*sev-wg*] eyes, most of the β-gal was found in the cone cells (data not shown). In the same focal plane as the ac-expressing cells, there is no overlap (Fig. 3D). Thus, the inhibitory effect of *wg* on *ac* expression is paracrine in nature.

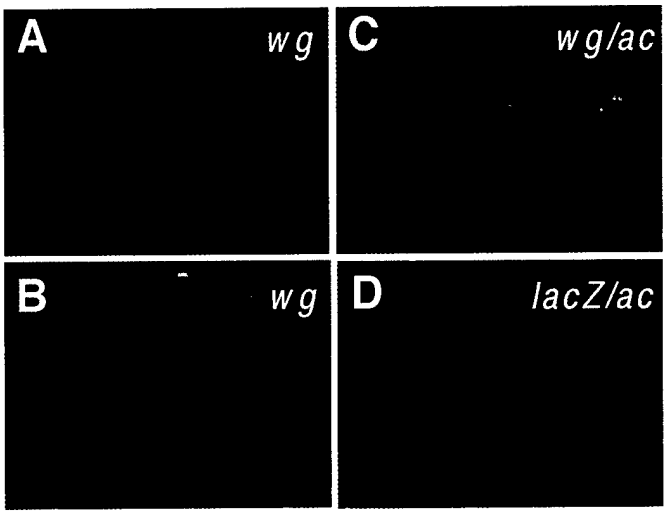

Fig. 3. The *sev* enhancer is not active in the ac-positive cells. P[*sev-wg*] (A-C) or P[*sev-lacZ*] (D) pupal eyes (all at 3 hours APF) were stained with antibodies against wg (A,B), wg and ac (C) or β-gal and ac (D) protein. All panels are confocal images, with *wg* and *lacZ* signals always in red and *ac* always in green. In control eyes, wg protein was detected in a ring around the periphery of the eye (data not shown), but no wg protein was detected in the eye proper. In P[*sev-wg*] eyes, wg is found primarily around the cone cells (A) and accumulated on their apical surface (B) but not in the few remaining ac-positive cells (C). The focal plane in C is about 15-20 μm basal of those in A and B. In the P[*sev-lacZ*] eyes (where *ac* expression is not affected) no β-gal protein is seen in the proneural clusters (D).

The *wg* signal transduction pathway in the eye

Extensive genetic analysis, confirmed by recent biochemical experiments, has identified four genes that encode probable components of the *wg* signaling pathway, *porc*, *dsh*, *zw3* and *arm* (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994; see introduction). Mosaic analysis (using the *w* gene as a marker) was performed to determine if these genes were required for the P[*sev-wg*]-dependent bristle inhibition. Control clones still lack bristles (Fig. 4A), as do clones mutant for the endogenous *wg* gene (Fig. 4C). In clones that lack the P[*sev-wg*] transgene, bristles are found almost to the clonal boundary (Fig. 4B). Likewise, 89% of the mutant clones for *porc*, *dsh* and *arm* had the full array of bristles within the clone (Fig. 4D-F and Table 1) and an additional 9% had a partial rescue of the bristleless phenotype. The remaining 2% that still lacked bristles were small in size and probably not completely mutant since the absence of the *w* gene cannot be detected on the surface of the eye at the cellular level. These experiments indicate that *porc*, *dsh* and *arm* are required for *wg*-dependent bristle inhibition.

zw3 is unique among the known genes required for *wg* signaling because it must be inhibited for the *wg* signal to be transduced (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Thus, loss of *zw3* should be equivalent to activation of *wg* signaling. Therefore, a *zw3* mutant clone in the eye might be expected to lack bristles. This straightforward analysis cannot be employed because the cells in *zw3* clones in the eye imaginal disc do not differentiate into eye tissue (Treisman and Rubin, 1995; data not shown). This is probably due to the fact that high levels of *wg* signaling activity prevent the morphogenetic furrow from progressing, blocking any subsequent differentiation (Treisman and Rubin, 1995).

If *zw3* must be inhibited for the *wg* signal to be transduced, then flooding cells with zw3 protein might titrate out the signal. This has been shown to be the case in *Xenopus* where overexpression of the homologue of zw3, glycogen-synthase kinase 3, blocks *Wnt* gene induction of dorsal mesoderm (Dominguez et al., 1995; He et al., 1995). We attempted a similar experiment by creating flies with one copy of P[*sev-wg*] (we chose one of the weaker P[*sev-wg*] lines, which at one copy has approximately 20 bristles/eye) and one or two copies of a heat-shock construct expressing the *zw3* gene, P[*hs-zw3*] (Siegfried et al., 1992). *zw3* was induced by heat shock shortly before and twice after the onset of pupation (see Fig. 5 legend for details). Though the results were not entirely conclusive (Fig. 5), many pupal eyes showed a significant response especially when the ratio of P[*hs-zw3*]/P[*sev-wg*] is two (Fig. 5C). Other heat-shock regimes were not as effective at suppressing the P[*sev-wg*] phenotype. These results are consistent with the current model for *zw3* function in *wg* signaling.

Overexpression of *dsh* has previously been found to mimic *wg* signaling in cultured cells (Yanagawa et al., 1995), frog embryos (Sokol et al. 1995; Rothbacher et al. 1995) and in the wing imaginal disc (Axelrod et al., 1996). The same P[*hs-dsh*] transgenic stock used in the wing can also duplicate the effect of *wg* in the eye. Induction of *dsh* at 3 hours (data not shown) or 6 hours APF (Fig. 6B) could block bristle formation, but heat shock at 9 hours APF (Fig. 6C) failed to inhibit bristles in the interior of the eye, though inhibition still occurred toward the periphery. This can be explained by previous work

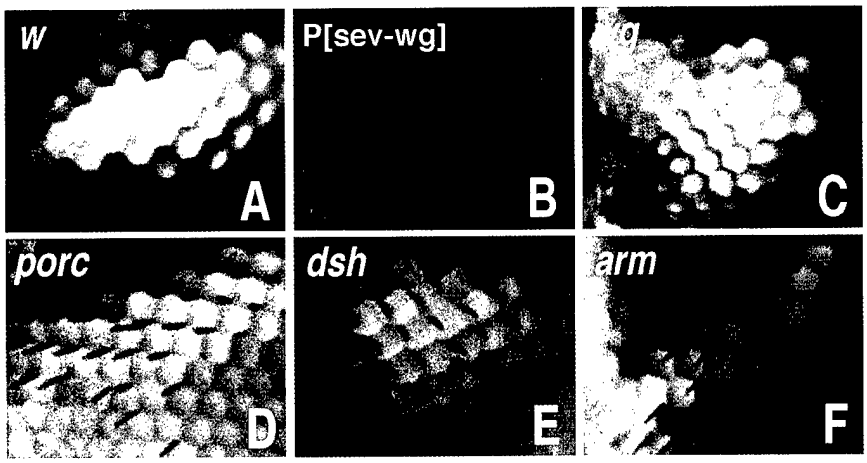

Fig. 4. The *porc*, *dsh* and *arm* genes are required for the P[*sev-wg*] phenotype, but the endogenous *wg* gene is not. Clones were induced in P[*sev-wg*] eyes as described in Materials and Methods. Clones were detected by the absence of pigmentation (from the *w* gene) in adult eyes. Bristles were still absent in control (A) or wg^{CX4} clones (C), but not in clones lacking the transgene (B) or homozygous for *porc* (D), *dsh* (E) and *arm* (F). A summary of all the data can be found in Table 1.

Table 1. Summary of the clonal analysis in a P[*sev-wg*] background (see Materials and Methods for details)

	Bristle density inside clone		
Chromosome	Bare	Partial	Full
P[*sev-wg*; w^+]	0	1	17
w	25	0	0
yw	21	1	0
wg^{CX4}	23	0	0
yw $porc^{2E}$	0	1	26
yw $porc^{I8}$	2	5	25
yw dsh^{477}	0	1	29
yw dsh^{V26}	1	4	14
w arm^{25B}	0	0	15
w arm^{XM19}	0	1	11

The P[*sev-wg*; w^+] clones are *w*;+/+ clones surrounded by *w*; P[*sev-wg*; w^+] tissue. The rest are clones of the homozygous genotype indicated and the entire eye, including the cells in the clone, are P[*sev-wg*; w^-]/+. Bare means no bristles found within the clone and full means the normal wild-type bristle density.

(Cagan and Ready, 1989a,b), which showed that SOP determination occurs first in the center of the eye and radiates outward concentrically. The same time requirements were seen when the bristles were inhibited using P[*hs-wg*] (data not shown).

Genetic and biochemical evidence places dsh downstream of wg in the signal transduction pathway (Klingensmith et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994; Yanagawa et al., 1995), suggesting that the overexpression of dsh can bypass wg function. However, in the wing, where dsh overexpression causes an expansion of the wing margin, it appears that *wg* gene activity is needed to see the *dsh* effect (Axelrod et al., 1996). In the eye, the opposite appears to be true. In pupa homozygous for a *wg* temperature-sensitive mutation, induction of *dsh* after 6 hours at the restrictive temperature still inhibited SOP formation (Fig. 6E). Thus it appears that dsh in the eye can act independently of wg, though caveats remain (see discussion).

The role of *N* in *wg* signaling in the eye

A strong interaction between mutations in the *N* and *wg* genes has been described (Couso and Martinez Arias, 1994; Hing et al., 1994), which suggests that the two genes have common developmental targets in some tissues. One report suggested that *wg* encodes a ligand for N, based on these genetic interactions and the fact that *N* encodes a transmembrane receptor-like protein (Couso and Martinez Arias, 1994). In the eye, *N* activity is required for almost every differentiated cell type (Cagan and Ready, 1989b), so examining *N* clones in a P[*sev-wg*] background was not possible. Therefore, we utilized N^{ts1}, a temperature-sensitive allele (Cagan and Ready, 1989b). When these flies were reared at the restrictive temperature for 3-11 hours APF in a P[*sev-wg*] background, a strong suppression of the *wg* bristleless phenotype was seen (Fig. 7B). This is consistent with a proposed role for *N* in transducing the *wg* signal. However, removal of *Dl* activity for the same time period also suppresses the P[*sev-wg*] phenotype (Fig. 7C).

N and Dl are key components in the lateral inhibition pathway (functioning as receptor and ligand, respectively) that insures the proper number of bristles in the eye (Cagan and Ready, 1989b; Parody and Muskavitch, 1993; note the abnormally high bristle density in Fig. 7B and C). This pathway is independent of *wg*, since mutant clones of *wg*, *porc*, *dsh* and *arm* in an otherwise wild-type background have the normal number of bristles (data not shown; see also Fig. 4D-F). Thus, the observation that loss of *Dl* activity can suppress the P[*sev-wg*] phenotype as well if not better than loss of *N* raises the possibility that the interaction between *N* and *wg* in the eye is due to the role of *N* in the lateral inhibition pathway.

If a higher level of *wg* expression is used (via a heat-shock promoter) all the bristles in the N^{ts1} background can be inhibited (data not shown; pupa were placed at the restrictive temperature for 6 hours before a 30 minute heat-shock pulse was given at 6 hours APF). However, it is known that the N^{ts1} allele does not completely remove *N* activity (Couso and Arias, 1994; Hartenstein et al., 1992) so this result is inconclusive. In the eye, it is not possible to determine whether wg works

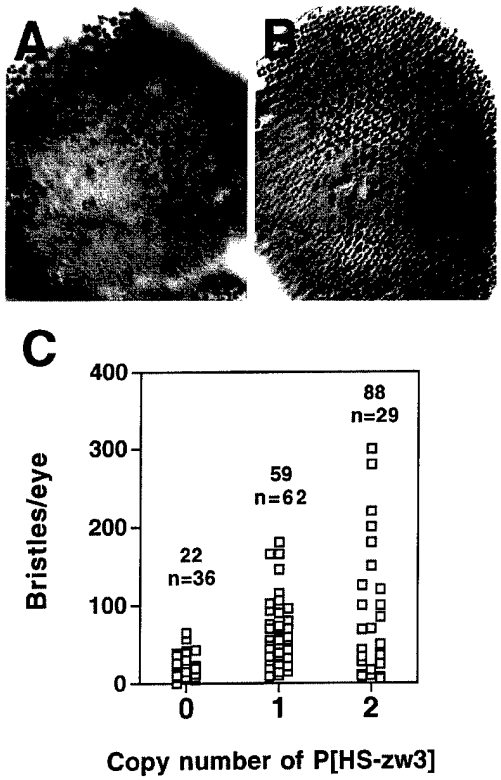

Fig. 5. Overexpression of *zw3* can suppress the P[*sev-wg*] phenotype. Three 1 hour heat shocks (37°C separated by two 4 hour recovery periods at 25°C) were given to animals containing one copy of P[*sev-wg*] and zero, one or two copies of P[*hs-zw3*] (the genotypes of the three groups were P[*sev-wg*; w^+]/+, P[*sev-wg*; w^+]/P[*hs-zw3*; w^+] and P[*sev-wg*; w^-], P[*hs-zw3*; w^+]/P[*hs-zw3*; w^+], respectively; all combinations were created from crosses of stocks described in Materials and Methods). The first heat shock was given at 1-2 hours prior to white pupa formation. An example of a control with about 25 SOPs (A) and a 1× P[*hs-zw3*] eye with about 180 SOPs (B) are shown. SOPs were detected with cut immunostaining. (C) The total data are summarized in a scatter plot. The mean number of SOPs are shown above each group, with the *n* value below. The standard deviation for the 0×, 1× and 2× groups were 15, 38 and 84, respectively. The differences between the 0× and the other two groups are significant at $P<0.001$ using a Student's *t*-test.

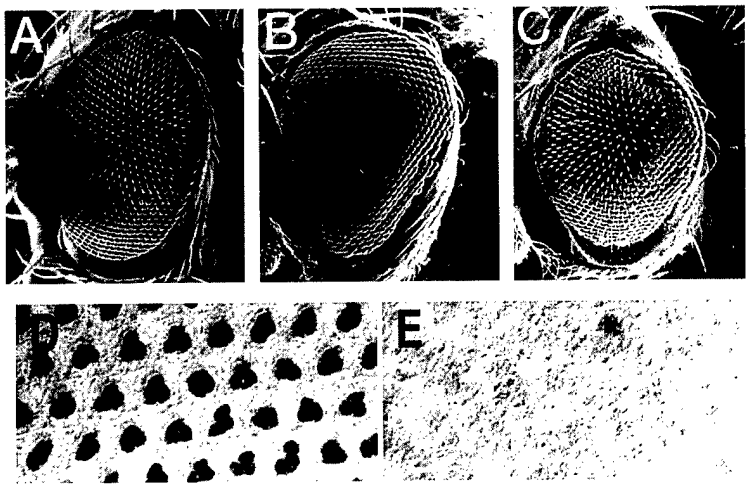

Fig. 6. Overexpression of *dsh* can inhibit bristle formation independently of *wg*. (A-C), SEM micrographs of P[*hs-dsh*] eyes given no heat shock (A) or a 30 minute heat shock (37°C) at 6 hours APF (B) or 9 hours APF (C). When *dsh* was induced at 6 hours APF, more than half the eyes had no or only a few bristles in the center of the eye (n=11) and the rest had a small patch of bristles in the center (n=8). At 9 hours APF, bristles were found over the interior two thirds of the eye but bristles were still missing toward the periphery (n=17). (D,E), cut stainings of P[*hs-dsh*], wg^{IL} homozygotes that were raised at 17°C (the permissive temperature) and then incubated at 29°C for 0-12 hours APF, without (D) or with (E) a 30 minute heat shock at 6 hours APF. Antibody stainings were done at ~30h APF. The cut-positive SOPs (now at the 4-cell stage) are completely absent in the heat shocked eyes (n=8). wg^{IL} homozygotes were identified as described in Materials and Methods.

through N or in a parallel pathway converging at proneural gene expression.

Role of *N* in *wg* signaling in the embryo

In order to more rigorously test the requirement of *N* for *wg* signaling, a tissue is needed where a putative *N-wg* connection can be separated from the *wg*-independent functions of *N*. One suitable place is the embryonic epidermis. Embryos mutant for *N* undergo a dramatic neural hyperplasia; almost all of the cells of the epidermis delaminate and become neuroblasts (Campos-Ortega, 1993). However, the epidermis remains relatively intact until full germ-band extension, after significant *wg* signaling has already occurred. Null *N* embryos were generated by making germ-line clones (Chou and Perrimon, 1992; see Materials and Methods). Antibody staining revealed no detectable N protein in *N* germline clones that have received a paternal Y chromosome (Fig. 8F). Thus we can examine *wg* signaling in a tissue that has never contained N protein.

Two well-characterized targets of *wg* signaling in the embryo are the *engrailed* (*en*) gene (DiNardo et al., 1988; Martinez-Arias et al., 1988) and the *wg* gene itself (Bejsovec and Wieschaus, 1993; Hooper, 1994; Yoffe et al., 1995). Careful analysis of expression of both genes has revealed that, in *wg* mutants, *wg* transcripts begin to fade before the embryo reaches full germ-band extension (stage 9; all stages according to (Campos-Ortega and Hartenstein, 1985), and is gone by the beginning of stage 10 (Manoukian et al., 1995). en protein in the adjacent posterior cells fades shortly thereafter. By mid-stage 10, both en protein and *wg* transcripts are completely gone from wg^{IN} homozygous embryos (Fig. 8B). In *N* null embryos at early stage 10, *wg* and *en* patterns are indistinguishable from wild type (data not shown). At mid-stage 10, both sets of stripes are still clearly present (Fig. 8C,D). The stripes do appear a little ragged, and we believe this is a consequence of the beginning of the disintegration of the epidermis, which is well underway by late stage 10 (about 15-20 minutes later than the embryos shown in Fig. 8).

Despite the results in Fig. 8, it might be argued that in *N* mutants, perhaps *wg* and *en* expression no longer depended on *wg* activity. To address this, we examined the affect of global *wg* expression on *en* transcript distribution in a *N* mutant background. As previously reported (Noordermeer et al., 1992, 1994), overexpression of *wg* via a heat-shock promoter in an otherwise wild-type background causes a dramatic posterior expansion of the *en* stripes so that they are about twice as wide as normal (compare Fig. 9A and B). This expansion in still seen in embryos lacking N protein (Fig. 9D) and is dependent on the presence of the P[*hs-wg*] transgene (Fig. 9C). In the complete absence of N protein, *wg* signaling appears normal as late as we can reliably assay for it.

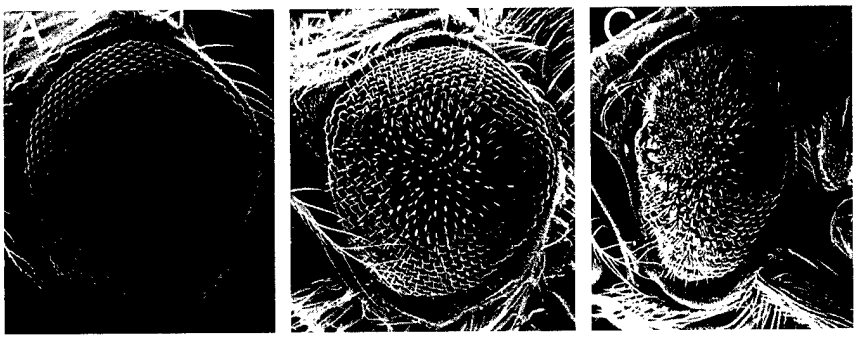

Fig. 7. Removal of *N* or *Dl* activity can suppress the P[*sev-wg*] bristleless phenotype. SEM micrographs of P[*sev-wg*]/+ (A), N^{ts1}/Y; P[*sev-wg*]/+ (B) and P[*sev-wg*], Dl^{RF}/Dl^{6E} (C) flies that were reared at 17°C and incubated at 32°C for 3 to 11 hours APF (7 hours APF at 17°C corresponds to 3 hours APF at 25°C) and then kept at 17°C until eclosion or dissection of pharates from pupal cases. Control and N^{ts1} hemizygotes were made by crossing P[*sev-wg*] males to either *w* or *w* N^{ts1} females. All males then had the desired genotype. P[*sev-wg*], Dl^{RF}/TM6C and Dl^{6E}/TM6C flies were crossed and appropriate animals identified by the absence of the dominant Tubby marker (found on TM6C). All N^{ts1} hemizygotes (n=20) and Dl^{RF}/Dl^{6E} transheterozygotes (n=9) showed the dramatic increase in bristle number. Note the higher than normal bristle density, indicative of the role these genes play in lateral inhibition. The *Dl* mutant combination consistently gave a more severe bristle hyperplasia than N^{ts1} in both a P[*sev-wg*] and non-transgenic background.

DISCUSSION

wg inhibits SOP formation at the level of the proneural genes

The interommatidial bristle is a 4-cell sensory organ that arises from a single SOP which is selected from a group of cells expressing proneural basic helix-loop-helix proteins (Campuzano and Modolell, 1992; Jan and Jan, 1993a). Our data strongly suggests that P[*sev-wg*]-derived wg protein blocks SOP formation in the eye by inhibiting proneural gene expression. Levels of ac protein are much lower in P[*sev-wg*] eyes (at 3 hours APF) compared to controls (Fig. 2C-F). 12 hours later, after the eye disc has everted, no SOP daughter cells are seen in the transgenic eyes (Fig. 2A,B). Though disc eversion prevents us from directly showing that no SOPs ever form in P[*sev-wg*] eyes, the time window when P[*hs-wg*] or P[*hs-dsh*] can inhibit bristle formation (no later than 6 hours APF for the central portion of the eye; Fig. 6 and results) is consistent with the model that, once an SOP is determined, *wg* signaling activity can no longer influence its fate.

The ac protein is the only proneural gene product monitored in this study and we are by no means suggesting that the *wg* signaling pathway acts directly on the *ac* promoter. In fact, lost of the *ac* gene alone does not result in complete elimination of interommatidial bristles; a related gene, *scute* (*sc*) must also be removed (Brown et al., 1991). The expression patterns of *ac* and *sc* are nearly identical (Cubas et al., 1991; Skeath and Carroll, 1991). This is most likely achieved by a combination of shared enhancer elements (Gómez-Skarmeta et al., 1995) and auto- and transactivation between the two genes (Martinez and Modolell, 1991; Skeath and Carroll, 1991; Van Doren et al., 1992). In addition, there are important negative inputs from other bHLH proteins such as *extramacrocheate* (Cubas and Modolell, 1992; Van Doren et al., 1992) and *hairy* (Brown et al., 1991; Van Doren et al., 1994). *wg* could be acting to inhibit *ac* (and presumably *sc*) expression at any of these regulatory levels. Further studies are needed to address this issue.

The P[*sev-wg*] bristleless phenotype was unexpected, because in the wing imaginal disc, *wg* has been shown to have the opposite effect, i.e., it promotes bristle development. In the absence of *wg* activity, the proneural ac-positive clusters fail to form (Couso et al., 1994; Phillips and Whittle, 1993). It is not clear why *wg* activates *ac* in one tissue and inhibits it in another, but this is a simple example of how one signal can generate different responses in various tissues.

wg is not normally expressed in the interior of the eye, but it is present at the periphery, forming a ring around the pupal eye (Cadigan and Nusse, unpublished data). Interestingly, the edge of the eye lacks bristles (Cagan and Ready, 1989b; Fig. 1A). Clones of *arm* at the periphery contain ectopic bristles (Cadigan and Nusse, unpublished data), suggesting that *wg* normally inhibits bristles there. However, large *wg* clones do not show this effect. We are currently examining this in more detail.

The *wg* signal transduction pathway in the eye

A genetic pathway for *wg* signal transduction has been elucidated in which the gene products work in the following order: *porc* → *wg* → *dsh* —| *zw3* —| *arm* (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Studies in the wing and leg imaginal disc have indicated that *dsh*, *zw3* and *arm* are also required there for *wg* signaling (Couso et al., 1994; Diaz-Benjumea and Cohen, 1994; Klingensmith et al., 1994; Peifer et al., 1991; Theisen et al., 1994; Wilder and Perrimon, 1995). This study extends these findings; *porc*, *dsh* and *arm* are clearly required for the ability of *wg* to inhibit eye bristles (Fig. 4; Table 1). The overexpression experiments with *zw3*, while not as conclusive (Fig. 5), are entirely consistent with the favored model, where *wg* acts by antagonizing *zw3* gene activity. While there may be exceptions (see below), it seems that most tissues use the same *wg* signaling components to achieve a variety of effects.

The mammalian counterpart of *zw3*, glycogen synthase kinase-3, has been shown to function in *ras*-dependent signaling (Stambolic and Woodgett, 1994). This raises the possibility that members of the *ras* and *wg* pathways share components in flies. In the eye, differentiation of photoreceptor cells is absolutely dependent on *ras*-dependent signaling (Simon et al., 1991). However, in clones of *dsh* and *arm*, all photoreceptors are present (S. Kaech, K.M. Cadigan and R. Nusse, unpublished observations). In the wing, clonal analysis with members of the *ras* pathway demonstrated that, unlike *wg*, they were not required for wing margin development (Diaz-Benjumea and Hafen, 1994). Thus, no interaction between these two pathways has yet been observed in *Drosophila*.

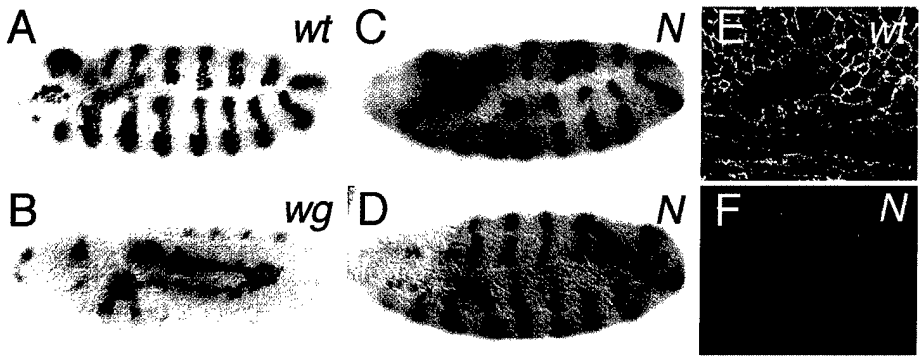

Fig. 8. *wg* signaling appears to be normal in *N* null mutant embryos. (A-D) Whole-mount staining for *wg* transcripts (blue) and/or en protein (brown) in wild-type (A), wg^{IN} (B) or N^{5419} (C,D) mutant embryos. All embryos are at mid-stage 10 (Campos-Ortega and Hartenstein, 1985). Both *wg* and *en* are absent at this stage from the epidermis of the *wg* mutants, but remain robust in the *N* mutant background (these embryos were also stained for β-gal protein, to unambiguously identify maternal and zygotic *N* mutants (see Material and Methods). (E,F) Confocal images of N antibody staining with a monoclonal antibody directed against the intracellular domain of N (Fehon et al., 1990) in N^{5419} germ-line clones receiving a paternal P[*ftz-lacZ*] (E) or Y (F) chromosome. N signal is completely lacking in the embryos that are negative for β-gal protein. Similar results in *wg*, *en* and *N* expression were obtained with a second *N* null allele, $N^{264.40}$ (data not shown).

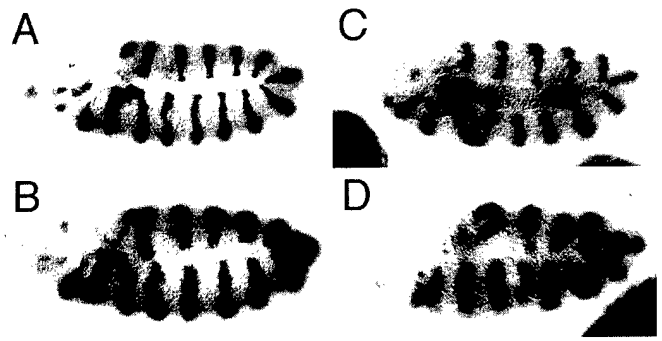

Fig. 9. The effect of ubiquitous expression of *wg* on *en* transcript distribution is still seen in a *N* null mutant background. All embryos are whole-mount stainings of *en* transcripts. (A) P[*hs-wg*] embryo with no heat shock. The *en* stripes are normal in appearance. (B) P[*hs-wg*] embryo after three 20 minute heat shocks (37°C) during early embryogenesis. The *en* stripes have expanded posteriorly, to about twice their normal width as previously described (Noordermeer et al., 1992). (C) N^{5419} null mutant after the three heat shocks. The stripes are somewhat ragged, but still present at the normal width. (D) N^{5419}; P[*hs-wg*] embryo after heat-shock treatment. The stripes have broadened as they do in a N^{+} background. All embryos were mid-stage 10 and the same results were obtained using the $N^{264.40}$ allele. *N* null embryos were created and identified as described in Materials and Methods.

wg expression is subject to positive autoregulation in the embryo (Bejsovec and Wieschaus, 1993; Hooper, 1994; Yoffe et al., 1995) and recent evidence suggests that this occurs through a distinct signaling mechanism (Hooper, 1994; Manoukian et al., 1995). Some discrepancies exist between the two reports, but Manoukian et al. (1995) provide strong evidence that *wg* autoregulation requires *porc* but not *dsh*, *zw3* and *arm*. They suggest a model where *porc* functions only in *wg* autoregulation and the other three genes in *wg* paracrine functions.

Our results in the eye indicate that, at least in the eye, *porc* is required for *wg* paracrine signaling. While we could clearly see *sev* enhancer-driven *wg* expression in cone cells and photoreceptors, we found no expression in the proneural clusters, the targets of *wg* action (Fig. 2). The endogenous *wg* gene was not required for the P[*sev-wg*]-dependent bristle inhibition (Fig. 4C), ruling out a paracrine-autocrine circuit. Our results indicating a role for *porc* in paracrine *wg* signaling are consistent with the observation that secretion or diffusion of wg protein is blocked in porc mutant embryos (Siegfried et al., 1994; van den Heuvel et al., 1993a).

Overexpression of *dsh* can mimic the action of *wg* in the eye (Fig. 6) as has been shown previously in the wing (Axelrod et al., 1996) and in cultured cells (Yanagawa et al., 1995). In the wing, this effect of *dsh* required *wg*. This does not appear to be the case in the eye (Fig. 6E). This is an important point because it speaks as to whether *dsh* can completely bypass the requirement for *wg* or whether overexpression of *dsh* simply potentiates *wg* signaling. It may be that there is residual *wg* activity left in our experiments (we could only rear the animals for 6 hours at the restrictive temperature before induction of *dsh*; longer times killed the organism before disc eversion). Another possibility is that a much higher threshold of *wg* activity is needed to transform wing blade to wing margin than is needed to inhibit eye bristles. The data of Axelrod et al. (1996) show that the transformation of identity is more penetrant closest to the normal wing margin, where *wg* is expressed. Thus, overexpression of *dsh* in the wing blade may not easily reach the necessary level of signaling to trigger the change in cell fate. In the eye, *dsh* is able (at 3 hours APF) to inhibit bristles in the middle of the eye (far from endogenous *wg* expression) just as efficiently as bristles closer to the periphery. That *dsh* can bypass the need for *wg* is also supported by the cell culture experiments (Yanagawa et al., 1995) where no detectable wg protein was observed under conditions where *dsh* could stabilize arm protein. In addition, Park et al. (1996) have recently shown that overexpression of *dsh* in the embryo can induce *wg* targets in a *wg* null background.

Is *N* required for *wg* signaling?

On the basis of genetic interactions between mutations in the two genes, the N protein was proposed to be a receptor (or part of a receptor complex) for wg (Couso and Martinez Arias, 1994). In the eye, we also observed strong genetic interactions between *wg* and *N* (Fig. 7). However, the interpretation of these experiments are complicated, since *N* is known to affect bristle development independently of *wg*, and because, for technical reasons, we could not completely remove *N* activity to determine whether *wg* signaling could still occur. Likewise, the previously published genetic interactions involve animals where *wg* and *N* activities are only partially removed (many of the experiments were done with double heterozygotes of various *wg* and *N* alleles), and are therefore subject to the same limits of interpretation.

Unlike the eye, *wg* signaling in the complete absence of *N* activity can be assayed in the embryonic epidermis until just after germ-band extension is complete (mid stage 10), right before the absence of *N* causes most of the epidermis to delaminate and become neuroblasts. We found no significant change in the expression of *wg* and *en* in *N* null mutants at this time (Fig. 8), even though their expression fades at early stage 10 in *wg* mutants and mutants in *dsh* or *arm* (Manoukian et al., 1995; Van den Heuvel et al., 1993b). In addition, the effect of overexpression of *wg* on the *en* stripes is still seen in a *N* mutant background (Fig. 9). Couso and Martinez Arias (1994) reported that the *en* stripes were affected in about half the *N* mutants they examined, but they used hyperplasia of the nervous system as their method for determining which embryos were *N* mutants. This happens after mid-stage 10, thus any effect on the stripes may be a secondary consequence of the epidermis falling apart. Therefore, we conclude that in *N* mutant embryos, *wg* signaling occurs normally, at least with regard to the two markers we assayed.

A similar conclusion with regards to *N-wg* interactions has been reached in the wing (Rulifson and Blair, 1995). They showed that *wg* could still regulate *ac* expression in homozygous clones for a *N* null allele. These mutant clones should completely lack *N*, barring prolonged perdurance of the N protein. Of equal importance is their finding that *N* activity is required for *wg* expression at the wing margin (see also Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996). This means that all of the genetic interactions between *wg* and *N* in the wing can potentially be explained by a reduction in *N* activity causing a reduction in the amount of *wg* signal, not the ability of *wg* to signal.

Another link between *wg* and *N* has been proposed by Axelrod et al. (1996), who have presented evidence that dsh protein can bind to and inhibit N activity in the wing imaginal disc. They suggest that part of the ability of *wg* to induce bristles in the wing is achieved by inhibition of *N* through *dsh*. Such an antagonistic relationship does not appear to be occuring in the eye since *wg*, *dsh* and *N* all inhibit bristle formation, although we can not rule out a mechanism where *wg* and *dsh* activate *N* to inhibit *ac* expression.

A subtle role for *N* in transducing the *wg* signal cannot be entirely ruled out. However, our results and those of Rulifson and Blair (1995) argue that in tissues where the direct test can be done, i.e., can *wg* signaling occur in cells that lack N protein, *N* is not required. A better candidate for a wg receptor is the product of the *Drosophila frizzled2* gene, which can bind to wg and tranduce the *wg* signal in cultured cells (Bhanot et al. 1996). N showed no activity in this wg-binding assay. In the absence of any biochemical data suggesting that the proteins interact, the simplest models for *wg* signal transduction should exclude a direct role for *N*.

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Cell Culture and Whole Animal Approaches to Understanding Signaling by Wnt Proteins in *Drosophila*

R. NUSSE, C. HARRYMAN SAMOS, M. BRINK, K. WILLERT, K.M. CADIGAN, A. WODARZ, M. FISH, AND E. RULIFSON

Howard Hughes Medical Institute, Department of Developmental Biology, Beckman Center, Stanford University Medical Center, Stanford, California 94305-5428

Wnt genes have essential roles in a wide variety of biological processes, ranging from mouse, frog, and fly embryogenesis to the initiation of cancer. This gene family includes the mouse mammary oncogene *Wnt-1* and its *Drosophila* ortholog *wingless* (*wg*), a segment polarity gene (Nusse and Varmus 1992; Klingensmith and Nusse 1994). Mutations in these genes have been described in many organisms, including mice and *Caenorhabditis elegans*. *Wnt* genes encode molecules equipped with a signal sequence, and the phenotypes caused by overexpression or loss-of-function mutations in *Wnt* genes suggested that their gene products are involved in cell-to-cell communication. Interestingly, in the *Drosophila* embryo and in imaginal discs, the Wg protein can work as a long-range patterning molecule, known as a morphogen (Lawrence et al. 1996; Zecca et al. 1996; Neumann and Cohen 1997). Until recently, however, very little was known about the biochemical mechanism of action of *Wnt* gene products. This was in part due to the problematic behavior of Wnt proteins in vitro (Papkoff and Schryver 1990) and the lack of a suitable cell culture assay for Wnt proteins. Over the past years, we have established various in vitro assays for signaling by Wnt proteins, based on the genetic analysis of wingless function in *Drosophila*. These assays have recently led to the identification of specific receptors for Wg and other Wnt proteins (Bhanot et al. 1996).

RESULTS AND DISCUSSION

Wg Signals through Armadillo

The basis of these in vitro assays is a key step in *wg* signaling in *Drosophila*: an increase in intracellular concentration of the Armadillo (Arm) protein. *wg* and *arm* are both members of the segment polarity class of mutations (Nüsslein-Volhard and Wieschaus 1980), sharing many phenotypic changes when mutated (Peifer et al. 1991). In the larval cuticle, absence of *wg* and *arm* causes a lawn of disoriented denticles. *wg* and *arm* are also required for the correct expression of a large set of target genes, including *engrailed* in the ectoderm. Clonal analysis of *arm* mutant cells shows that *arm* is cell autonomous (Wieschaus and Riggleman 1987), whereas *wg* is nonautonomous, suggesting that *arm* functions downstream from *wg*. By epistasis experiments, using various combinations of gain-of-function and loss-of-function alleles of members of the *wg* signaling pathway, we and others have shown that Arm is required for transduction of the Wg signal to downstream events, including the expression of *engrailed* (Noordermeer et al. 1994; Peifer et al. 1994; Siegfried et al. 1994).

In addition to being required for Wg signal transduction, Arm protein levels are regulated by *wg*. Staining of wild-type *Drosophila* embryos with antibodies to Arm gives a strong signal in areas in which Wg is expressed, and this stronger staining is absent in *wg* mutants (Riggleman et al. 1990). Conversely, ubiquitous expression of Wg, under the control of a heat shock promoter, leads to relatively uniform intense staining for Arm (Noordermeer et al. 1992). As Arm mRNA levels are not influenced by Wg, the change in the Arm protein seems to be at the posttranscriptional level.

Recently, various groups have provided evidence for a novel and interesting mechanism of action of Arm as a signal transduction component. The product of *arm* is similar to the mammalian proteins β-catenin and plakoglobin, which are present in junctional complexes. Arm was therefore thought to function in cell adhesion rather than in relaying a signal to the nucleus, but surprisingly, Arm also can act as an activator of transcription. Arm is in itself not a DNA-binding protein and, under normal circumstances, is not found in the nucleus. Arm can bind, however, to a DNA-binding protein related to the mammalian Lef-1 or TCF proteins (Behrens et al. 1996; Molenaar et al. 1996). These proteins contain an HMG box and were found originally as enhancer binding factors for T-cell-specific genes (Clevers and Grosschedl 1996). A *Drosophila* homolog of TCF-1, also named *pangolin*, has been cloned and specific mutations in the gene display a segment polarity phenotype (Brunner et al. 1997; van de Wetering et al. 1997). Moreover, *TCF/pangolin* is genetically downstream from Arm: The effect of a constitutively active form of *Arm*, lacking part of the amino terminus, is blocked by the absence of TCF (for review, see Nusse 1997).

Additional epistasis experiments in *Drosophila* have led to a genetic outline of Wg signaling (Fig. 1), in which

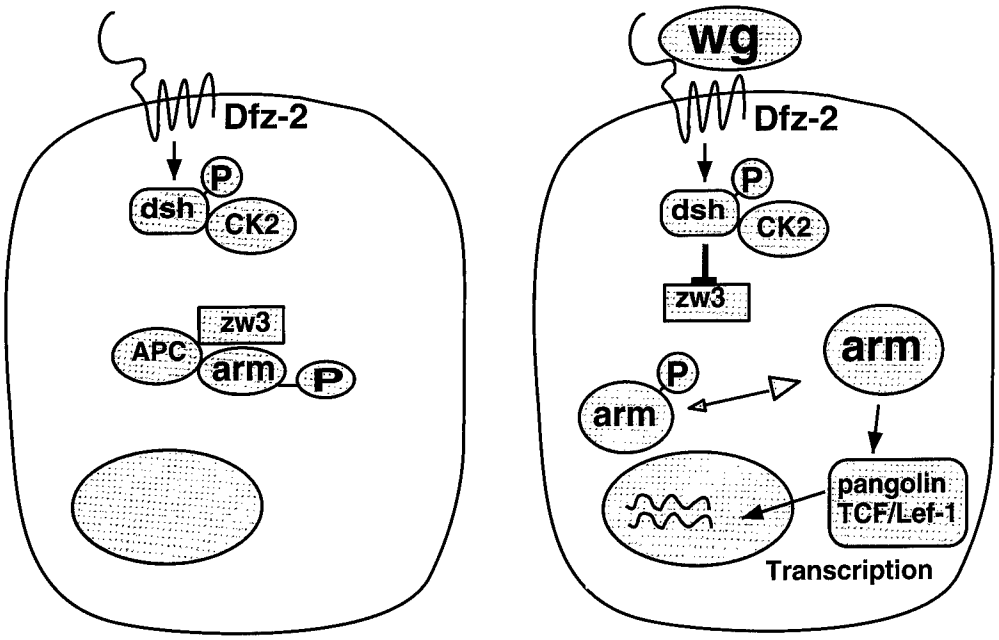


Figure 1. The Wg signal transduction pathway, as derived from genetic and biochemical analysis. In the absence of Wg (*left*), the zeste white 3 (zw3) protein kinase, the Armadillo (Arm), and the APC protein form a complex (Rubinfeld et al. 1996) that results in the down-regulation of Arm. In cells activated by Wg, the signal is transduced by the Dfz2 receptor (Bhanot et al. 1996) (or other frizzled family members) through the Dishevelled (Dsh) protein. Dsh is phosphorylated by CK2 (Willert et al. 1997), a phosphorylation step that is controlled by Dfz, possibly followed by other kinases. The Wg signal results in the inactivation of zw3 (or the effects of this kinase) and up-regulation of the Arm protein. Arm can now bind to members of the TCF/Pangolin/Lef-1 family (Brunner et al. 1997; van de Wetering et al. 1997) and is translocated to the nucleus, where it controls gene expression.

Wg acts through the dishevelled gene (*dsh*) to counteract the activity of a protein kinase zw3. In the absence of Wg signaling, zw3 inactivates Arm, possibly by direct phosphorylation of Arm followed by proteolytic breakdown. This effect of zw3 on Arm is then relieved by *wg*, resulting in stabilization and up-regulation of the Arm protein. Genetic studies in *Drosophila* have not revealed how Wg would interact with a specific cell surface receptor.

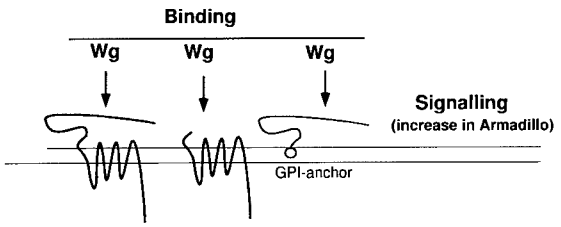
Wg Can Activate Arm in a Cell Line

Using a cell line (clone-8) derived from *Drosophila* imaginal discs, we found that Wg significantly elevates the concentration of the Arm protein in cell culture as well (Van Leeuwen et al. 1994). By transfecting the cells with a temperature-sensitive allele of Wg, we found that the accumulation is due to an increased stability of the Arm protein. By pulse-chase experiments, we could show that Arm has a rapid turnover in the absence of Wg. Wg stabilizes Arm, possibly by inactivating the protein kinase activity of zw3, as suggested by the genetic interactions and the evidence that the mammalian homolog of zw3, GSK3, can be down-regulated by Wg in certain cell lines (Cook et al. 1996).

We could also use this fast and quantitative effect to demonstrate, for the first time, that active Wg protein is present in the extracellular matrix (ECM) and in soluble form in the medium (Van Leeuwen et al. 1994). We concluded that clone-8 cells are very sensitive to the Wg protein, perhaps because they would express a specific Wg receptor. Similar experiments done with *Drosophila* S2 cells showed that these cells were unable to respond to Wg protein (Yanagawa et al. 1995).

In Vitro, Members of the Frizzled Protein Family Can Act as Receptors for Wg and Other Wnt Proteins

Frizzled (Fz) proteins are part of the large family of seven membrane-spanning domain receptors (sometimes referred to as serpentine receptors). In *Drosophila*, this gene family counts three members, so far as known. The original *frizzled (fz)* gene was identified through its tissue polarity phenotype, generating disoriented cells in the wing blade (Vinson and Adler 1987; Vinson et al. 1989). Although *fz* is expressed in the *Drosophila* embryo, it appears not to be essential for early embryogenesis. In addition, the *smoothened* (*smo*) gene, which is implicated in *hedgehog* (*hh*) signal transduction, is a *fz* family member (Alcedo et al. 1996; Van Den Heuvel and Ingham 1996). The Hh protein uses Smo to transduce the signal, but binds primarily to another multiple transmembrane protein, Patched (Marigo et al. 1996; Stone et al. 1996). Finally, a *Drosophila* frizzled-related gene, D*fz*2, is expressed in the embryo in a pattern reminiscent of some segment polarity genes, such as Wg (Bhanot et al. 1996). We found that D*fz*2 is expressed in a *Drosophila* clone-8 cell line that is *wg*-responsive, but not in nonresponding S2 cells (the assay for *wg* activity being the stabilization and subsequent accumulation of the Arm). After transfection with the D*fz*2 gene, S2 cells are able to transduce the *wg* signal. In addition, the S2 cells can now bind Wg



fz	+		+
Dfz2	+		+
Dfz2δ(CRD)		–	–
Dfz2ED-GPI		+	–
smo	–		–
Mfz3	–		–
Mfz4	+		+
Mfz6	–		nt
Mfz8	+		+
Hfz5	+		+

Figure 2. Wg protein binding to, and signal transduction by, several members of the frizzled gene family and mutant forms. Wg binds to full-length frizzled, Defrizzled-2 (D*fz*2), the GPI-linked extracellular domain of D*fz*2 (Bhanot et al. 1996), and several mammalian *fz* genes, as indicated. Binding to the full-length forms leads to signaling in S2 cells transfected with these Fz forms, as the levels of the Arm protein are increased by Wg in a receptor-dependent manner (*right side*). Fz variants that do not bind Wg (smoothened, D*fz*2 without the extracellular domain, and mouse Fz3 and mouse Fz6) do not elevate Arm either. The GPI-linked extracellular domain of D*fz*2 binds Wg but is unable to transduce the signal.

protein on their cell surface (Bhanot et al. 1996). Transfection of cells with D*fz*2 constructs lacking either the extracellular or intracellular domain of the protein demonstrated that the extracellular domain was required for binding (Fig. 2). Although a direct interaction between the Wg protein and D*fz*2 is still lacking, the data suggest that D*fz*2 can bind to and transduce the Wg signal. The Smo protein, when tested in the same assay, does not bind Wg, but of the identified mouse and human Fz proteins (Y. Wang et al. 1996, 1997b), several are also positive in the Wg binding assay described above (see Fig. 2; *smo* clone kindly provided by Marcel Van Den Heuvel and Phil Ingham). There is a correlation between the sequence distances between these various Fz family members and their capacity to bind Wg, because the receptors that are more distantly related to D*fz*2, including Smo, do not bind (Fig. 3). Presumably, these Fz family members bind other Wnt proteins.

We have also tested for signal transduction, by transfection of Fz constructs into S2 cells and measuring the Arm protein concentration before and after adding soluble Wg protein. Without exception, the Fz proteins that were able to bind Wg did also transduce the Wg signal to Arm (see Fig. 2). The original *fz* gene can confer Wg responsiveness to nonresponding cultured cells, as well as Wg binding. One possibility is that *fz* acts redundantly with D*fz*2 or other as yet unidentified frizzled proteins to transduce the *wg* signal in vivo. However, it does appear likely that some *Drosophila Wnt* gene is the ligand for the polarity function of *fz*.

Other Evidence for Wnt-frizzled Interactions

The results mentioned above, and the fact that there are no D*fz*2 mutants, make it uncertain whether D*fz*2 is the physiological *wg* receptor. We have obtained some evidence that D*fz*2 can act as a receptor for Wg by expressing the extracellular domain of the protein as a GPI-linked cell surface protein in *Drosophila* imaginal discs, using the GAL-4/UAS system. The wings of the resulting flies have marked defects in the margin (Fig. 4), known to be specified by Wg, and in other structures such as the eyes and the legs (data not shown). All the phenotypes observed are similar to loss of Wg function, which suggests that the extracellular domain binds Wg and inhibits its function.

The proposal of Fz molecules functioning as Wnt receptors was strengthened by recent work from nematodes and frogs. In the nematode *C. elegans*, there are two genes involved in asymmetric cell divisions of certain cell lineages. One of these genes (*lin-44*) encodes a *Wnt*

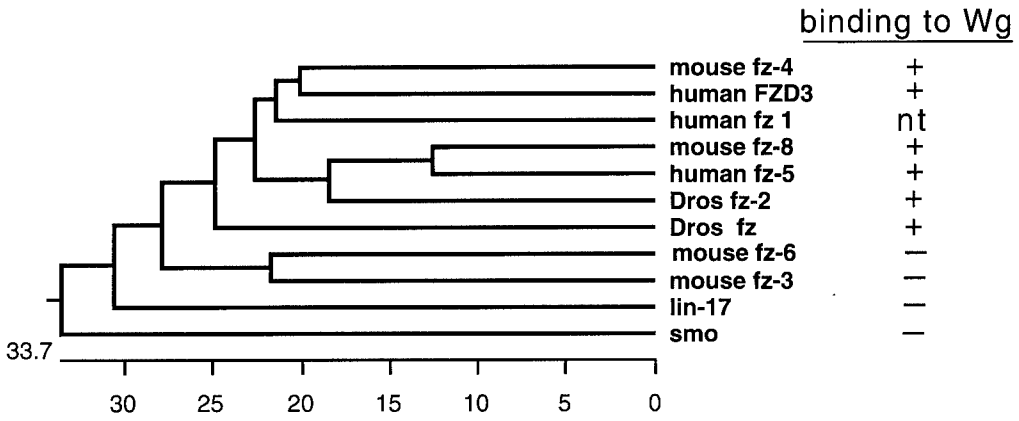


Figure 3. Sequence distances between various *fz* family members and binding to Wg protein. A dendrogram (produced by the DNA Star Software suite, Lasergene Inc.) shows the evolutionary distance between Fz protein sequences. Fz proteins closely related to D*fz*2 bind Wg, but more distantly related proteins (mfz3, mfz6, lin-17 and smo) do not.

Figure 4. Effects of overexpression of the GPI-linked D*fz*2 extracellular domain in the *Drosophila* wing. The GPI-linked D*fz*2 extracellular domain was expressed using the Gal-4/UAS system. This results in loss of the wing margin, which is also a phenotype caused by loss of *wg* function (Couso et al. 1993).

gene (Herman et al. 1995), the other, *lin-17*, encodes a *frizzled* family member (Sawa et al. 1996). The phenotypes of the two mutants are similar but not identical, but the biochemical evidence summarized above suggests a ligand-receptor relationship for these genes as well.

In *Xenopus* embryos, injection of Wnt RNA has long been known to induce a secondary body axis. Some *Wnt* genes lack this activity; it has been suggested that they function through a different signaling mechanism (Christian et al. 1991). If these Wnts are coinjected with the appropriate frizzled gene's RNA, the axis duplication effect is restored (He et al. 1997). This suggests that the only difference between the two classes of frog *Wnt* genes may be their affinity for the endogenous Fz receptor. More recently, secreted forms of Fz proteins have been found in *Xenopus* and in other vertebrates (Finch et al. 1997; Leyns et al. 1997; Rattner et al. 1997; S.W. Wang et al. 1997). These proteins, called FRP or Frzb, are made by the Spemann organizer and counteract the ventralizing activity of Xwnt-8 during frog embryogenesis (Leyns et al. 1997; S.W. Wang et al. 1997).

Function of Dishevelled in Wg Signaling

On the basis of genetic analysis of the *wg* signal transduction pathway, *wg* activates *dsh*, which in turn inhibits *zw3*. *dsh* encodes a cytoplasmic protein (Dsh) with no known biochemical function and little homology with other proteins (Klingensmith et al. 1994; Theisen et al. 1994). Several *dsh* genes (Dvl-1, 2, and 3) have been cloned from the mouse by homology (Sussman et al. 1994). Sequence comparison of *dsh* genes reveals regions of high homology in the amino terminus and in the central domain, whereas the carboxyl terminus is divergent (Fig. 5). The central region of Dsh contains a PDZ domain (Fig. 5) (Ponting 1995). Structural analysis has shown that a carboxy-terminal four-residue motif (X-Thr/Ser-X-Val) binds to the PDZ domain of a number of

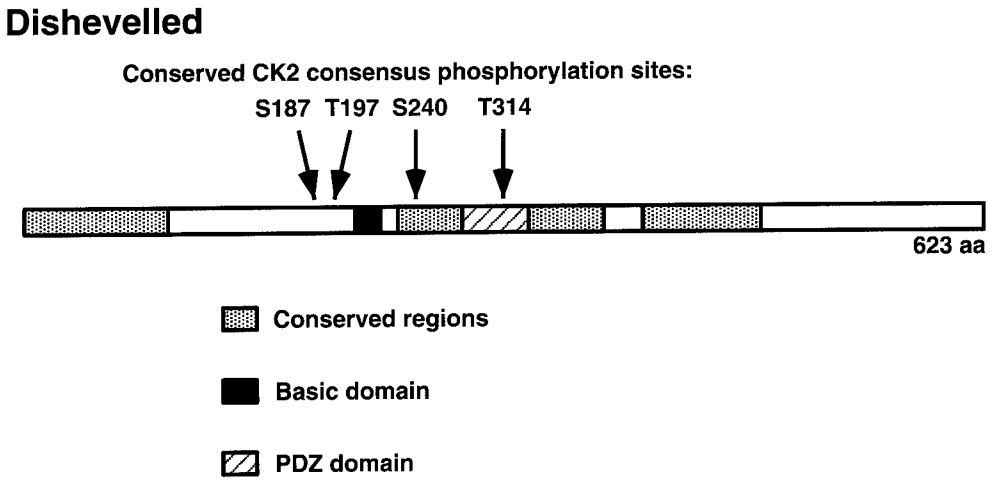


Figure 5. Schematic structure of the Dishevelled protein, with CK2 consensus substrate sites (Willert et al. 1997). Dsh has various highly conserved domains, including a DEP and a PDZ domain. CK2 sites are found at various positions, including one in the PDZ domain.

proteins (Doyle et al. 1996). However, no ligand for the PDZ domain of Dsh has yet been identified. Because several proteins have a similar carboxy-terminal motif as well, we have tested whether Dsh binds to D*fz*2, but these assays have failed to provide evidence that this is the case (data not shown).

The carboxyl side of Dsh contains a recently described domain referred to as the DEP domain (Ponting 1996). DEP domains are found in a variety of proteins many of which participate in G-protein signaling. Directly amino-terminal to the PDZ of Dsh is a conserved stretch of basic residues that, in many other proteins, serves as a nuclear localization signal (Fig. 5). However, cell biological studies have shown that Dsh is localized predominantly in the cytoplasm of the cell and not in the nucleus (Yanagawa et al. 1995). The functions of the PDZ, DEP, and basic domains in Dsh are unknown.

To test for possible biochemical functions of Dsh, we examined the protein in cells stimulated by Wg. Stimulation of the wing imaginal disc cell line clone 8 with Wg-conditioned medium leads to the accumulation of Arm and the hyperphosphorylation of Dsh (Yanagawa et al. 1995). This suggested that the hyperphosphorylated form of Dsh is the active form of Dsh. We were therefore interested to see that a kinase activity is associated with Dsh protein in cultured cells and in embryos and that this kinase phosphorylates *Dsh* in vitro. When Dsh protein is immunoprecipitated from those cells and the immune complex is subjected to an in vitro kinase reaction, it becomes phosphorylated (Willert et al. 1997). Phospho amino acid analysis of Dsh showed that most of the phosphorylation occurs on serine, some on threonine, and none on tyrosine.

We used the specific binding of the kinase to Dsh as a first step in the purification of this enzyme. A soluble protein extract from S2 *dsh* cells was applied to an anti-Dsh antibody affinity column and fractions were assayed for kinase activity toward a Dsh fusion protein. This resulted in two bands, of 38 and 28 kD, which were isolated and subjected to peptide sequencing. One tryptic peptide was sequenced, and the amino acid sequence revealed a perfect match with the α-subunit of *Drosophila melanogaster* caseine kinase 2 (CK2). We also found CK2 to coimmunoprecipitate with Dsh in lysates prepared from *Drosophila* embryos, demonstrating that CK2 is associated with Dsh in the *Drosophila* embryo (Willert et al. 1997). Dsh has a number of CK2 consensus phosphorylation sites (Fig. 5), and we are in the process of inactivating these sites to examine possible consequences for Dsh activity.

CK2 Is Associated with Dsh in Cells Overexpressing D*fz*2

Interestingly, we found a difference in CK2 binding to Dsh when we compared normal and D*fz*2-transfected S2 cells (Willert et al. 1997). In parental S2 cells, which do not express D*fz*2, Dsh migrates as a single band of 70 kD. Uninduced S2 D*fz*2 cells contain low, but detectable, levels of D*fz*2 protein and display a series of Dsh bands that migrate more slowly than Dsh in S2 cells. Clone-8 cells, which normally express D*fz*2, also contain a series of differently phosphorylated forms (data not shown). Unlike overexpression of Dsh, which leads to the accumulation of Arm protein, overexpression of D*fz*2 is not sufficient to increase Arm. Apparently, the phosphorylation of Dsh caused by D*fz*2 overexpression is not sufficient for the transduction of the signal to Arm. These findings point to complicated interactions between the various components of Wg signaling, to be addressed by further genetic and biochemical studies.

ACKNOWLEDGMENTS

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Casein kinase 2 associates with and phosphorylates Dishevelled

Karl Willert[1,2], Marcel Brink[1], Andreas Wodarz[1], Harold Varmus[3] and Roel Nusse[1,4]

[1]Howard Hughes Medical Institute and Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, [2]Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143 and [3]National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

[4]Corresponding author

The *dishevelled* (*dsh*) gene of *Drosophila melanogaster* encodes a phosphoprotein whose phosphorylation state is elevated by Wingless stimulation, suggesting that the phosphorylation of Dsh and the kinase(s) responsible for this phosphorylation are integral parts of the Wg signaling pathway. We found that immunoprecipitated Dsh protein from embryos and from cells in tissue culture is associated with a kinase activity that phosphorylates Dsh *in vitro*. Purification and peptide sequencing of a 38 kDa protein co-purifying with this kinase activity showed it to be identical to *Drosophila* Casein Kinase 2 (CK2). Tryptic phosphopeptide mapping indicates that identical peptides are phosphorylated by CK2 *in vitro* and *in vivo*, suggesting that CK2 is at least one of the kinases that phosphorylates Dsh. Overexpression of *Dfz2*, a Wingless receptor, also stimulated phosphorylation of Dsh, Dsh-associated kinase activity, and association of CK2 with Dsh, thus suggesting a role for CK2 in the transduction of the Wg signal.

Keywords: casein kinase 2/Dishevelled/Frizzled/Wingless/Wnt

Introduction

The *Drosophila* segment polarity gene *wingless* (*wg*) is a member of the *Wnt* gene family, which encodes secreted glycoproteins with diverse functions in developmental processes (reviewed in Nusse and Varmus, 1992). The biochemical analysis of the signal transduction pathway of the Wingless protein (Wg) has made significant progress in recent years with the development of an *in vitro* assay for soluble Wg protein (van Leeuwen *et al.*, 1994) and the identification of *Drosophila frizzled 2* (*Dfz2*) as a receptor for Wg (Bhanot *et al.*, 1996).

Genetic analysis of *wg* has led to the identification of several components required for the transduction of the Wg signal. Among these signal transducing molecules are the protein products of the segment polarity genes *dishevelled* (*dsh*), *zeste-white3* (*zw3*) and *armadillo* (*arm*). Genetic epistasis experiments have ordered these genes into a linear pathway that has served as a guide for the biochemical dissection of the Wg signal transduction pathway (reviewed in Nusse and Varmus, 1992; Klingensmith and Nusse, 1994; Perrimon, 1994). In this pathway, *wg* activates *dsh* which in turn inhibits *zw3*. Inhibited *zw3* no longer inhibits *arm*, so that the net effect of *wg* signaling is the activation of *arm*.

Dsh encodes a cytoplasmic protein (Dsh) with no known biochemical function and little homology to other proteins (Klingensmith *et al.*, 1994). In the mouse, three *dsh* genes (*Dvl-1*, *2* and *3*) have been cloned by homology (Sussman *et al.*, 1994). Sequence comparison of *dsh* genes reveals regions of high homology in the amino-terminus and in the central domain while the carboxy-terminus is highly divergent (Klingensmith *et al.*, 1994). The central region of Dsh contains a domain referred to as *discs-large* homology region (Dhr) or PDZ (Klingensmith *et al.*, 1994; Ponting, 1995), which is found in several other proteins, such as PSD-95, ZO-1 and Discs-large (Ponting, 1995). Structural analysis demonstrated that a carboxy-terminal four residue motif (X-Thr/Ser-X-Val) binds to the PDZ domain of PSD-95 (Doyle *et al.*, 1996) and to the PDZ domain of the human homolog of the *Drosophila discs-large* tumor suppressor gene product, DlgA (Morais Cabral *et al.*, 1996). However, no ligand for the PDZ domain of Dsh has been identified yet. On the carboxyl side of the PDZ domain in Dsh is a recently described domain referred to as the DEP domain (Ponting, 1996). DEP domains are found in a variety of proteins, many of which participate in G-protein signaling. Directly amino terminal to the PDZ of Dsh is a conserved stretch of basic residues which in many other proteins serves as a nuclear localization signal. However, cell biological studies have shown that Dsh is localized predominately in the cytoplasm of the cell (Yanagawa *et al.*, 1995) and not in the nucleus. The functions of the PDZ, DEP and basic domains in Dsh still need to be elucidated.

Stimulation of the wing imaginal disc cell line Clone 8 (Cl8) with Wg-conditioned medium leads to the accumulation of Armadillo protein (Arm; van Leeuwen *et al.*, 1994) and the hyperphosphorylation of Dsh (Yanagawa *et al.*, 1995). Since genetic analysis of the *wg* gene suggests that the Wg signal activates the Dsh protein (reviewed in Nusse and Varmus, 1992; Perrimon, 1994), we hypothesized that the hyperphosphorylated form of Dsh is the active form of Dsh (Yanagawa *et al.*, 1995). In this model, Wg stimulates the phosphorylation of Dsh, and phosphorylated Dsh transduces the signal onto the next signaling component, directly or indirectly leading to the inhibition of Zeste-white 3.

In this paper, we show that a kinase activity is associated with Dsh protein in cultured cells and in embryos, and that this kinase phosphorylates Dsh *in vitro*. We purified this protein kinase and demonstrated it to be identical to Casein Kinase 2 (CK2). Tryptic phosphopeptide mapping strongly suggests that CK2 phosphorylates Dsh both

in vitro and *in vivo*. The phosphorylation of Dsh is regulated by expression of *Dfz2*, a gene encoding a Wg receptor (Bhanot *et al.*, 1996). These results demonstrate that CK2 phosphorylates Dsh in response to *Dsh* and *Dfz2* expression and suggest a role for CK2 in Wg signaling.

Results

Characterization of a Dsh-associated kinase

As demonstrated previously, treatment of the wing imaginal disc cell line Clone 8 with Wingless protein leads to the phosphorylation of the Dsh protein (Yanagawa *et al.*, 1995). Furthermore, this phosphorylation can be promoted by overexpression of the Dsh protein itself (Yanagawa *et al.*, 1995).

To identify a kinase responsible for the phosphorylation of Dsh, we asked whether a kinase activity was associated with Dsh. Schneider 2 (S2) cells were transfected with a *dsh* cDNA under the control of the metallothionein promoter (S2*dsh*). *Dsh* overexpression can be induced in S2*dsh* cells by the addition of copper ions (Cu^{2+}). Untransfected S2 cells display a single band of Dsh protein, while transfection of *dsh* into S2 cells leads to a lower mobility of the Dsh protein band (Figure 1A, top panel), an effect due to the phosphorylation of Dsh. Further overexpression of Dsh by incubating the cells in Cu^{2+} leads to an additional shift in mobility due to further phosphorylation of Dsh. As shown previously (Yanagawa *et al.*, 1995), overexpression of *dsh* also leads to the accumulation of the Armadillo (Arm) protein (Figure 1A, bottom panel).

When Dsh protein is immunoprecipitated from S2*dsh* cells induced to overexpress *dsh* ($+Cu^{2+}$) and the immune complex is subjected to an *in vitro* kinase reaction, a protein with the molecular weight of 70 kDa (the size of Dsh) becomes phosphorylated (Figure 1A, middle panel). *In vitro* kinase reactions on Dsh immunoprecipitates from parental S2 cells and uninduced S2*dsh* ($-Cu^{2+}$) cells do not display this predominant phosphoprotein. This kinase activity also co-immunoprecipitated with Dsh from embryo lysates (Figure 1A), and from cell lysates of the wing imaginal disc cell line, Clone 8, overexpressing the *dsh* gene tagged with a myc epitope (Figure 1B).

When a carboxy-terminally deleted Dsh protein, ΔC-term Dsh (Yanagawa *et al.*, 1995), is overexpressed and subjected to the *in vitro* kinase reaction described above, the detected phosphorylated protein is smaller (Figure 1B), corresponding to the reduction in molecular weight of ΔC-term Dsh relative to wild-type Dsh. This demonstrates that the phosphoprotein produced in the *in vitro* kinase reaction is Dsh itself.

The kinase activity present in Dsh immunoprecipitates may be due either to a Dsh-associated kinase or to an intrinsic kinase activity of Dsh. Since Dsh is not related to any protein kinases on the basis of amino acid sequence, it is likely that the kinase activity is associated with Dsh, rather than Dsh itself. To address this, we attempted to disrupt and reconstitute the kinase activity by changing the ionic strength of the buffer in the immunoprecipitation. When immunoprecipitated Dsh protein was washed with a high salt buffer and then subjected to an *in vitro* kinase reaction, much of the kinase activity was removed from the complex (Figure 2A, lane 2). The kinase activity on Dsh could be restored by diluting the high salt wash

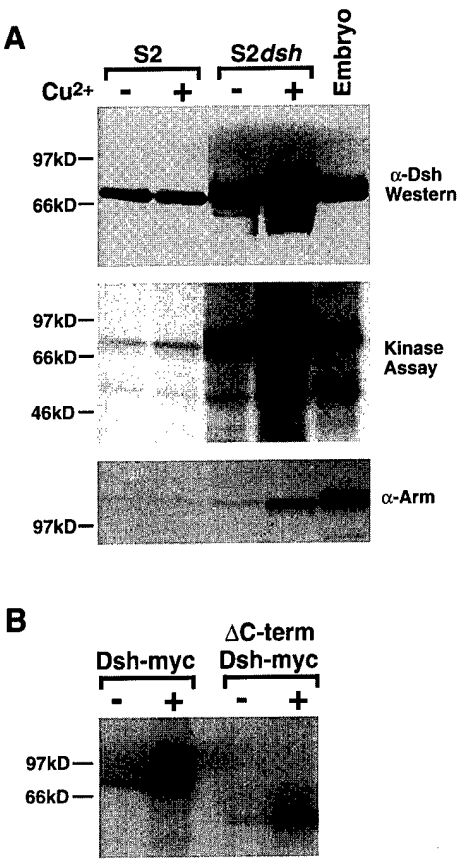

Fig. 1. A Dsh specific kinase activity is associated with Dsh. (**A**) A kinase activity co-immunoprecipitates with Dsh. S2 and S2*dsh* cells were either treated (+) or not treated (–) with 0.5 mM $CuSO_4$ to modulate expression of the transfected Dsh gene. Equal amounts of total cell lysates from these cells and from embryos were immunoblotted with anti-Dsh (top panel) or anti-Arm antibody (bottom panel). Dsh protein was immunoprecipitated from equal amounts of total cell extracts of S2, S2*dsh* cells and embryos with anti-Dsh antibody, and the immune complexes were incubated with [γ-^{32}P]ATP for an *in vitro* kinase reaction (middle panel). A longer exposure of the anti-Arm blot reveals the presence of equal amounts of Arm protein in S2 and in uninduced S2*dsh* cells. (**B**) The kinase activity co-immunoprecipitating with Dsh phosphorylates Dsh. Dsh was immunoprecipitated from lysates of either Clone 8 *dsh-myc* or Clone 8 *ΔC-term dsh-myc* cell lysates with anti-Myc antibody (9E10) and the immune complexes (Dsh-myc and ΔC-term Dsh-myc) were used in *in vitro* kinase reactions. Cells were either treated (+) or not treated (–) with 0.5 mM $CuSO_4$ to modulate expression of the transfected genes.

to lower salt concentrations and adding it back to the immunoprecipitated Dsh protein (Figure 2A, lane 3). Thus, the protein kinase activity can be specifically eluted from Dsh and reconstituted on Dsh.

To determine which domain(s) of Dsh are phosphorylated, a series of deletions in Dsh were constructed. Bacterially expressed fusion proteins between glutathione *S*-transferase (GST) and various fragments of Dsh were purified, incubated with the eluted Dsh-specific kinase activity, and subjected to an *in vitro* kinase reaction. Full-length Dsh fused to GST (Full) was efficiently phosphorylated (Figure 2B). GST fusions with either the amino- or the carboxy-terminus of Dsh (G1 and G12) or GST alone failed to become significantly phosphorylated while GST fusions with the central domain of Dsh (G4, G5, G7) were efficiently phosphorylated. Thus, the central domain,

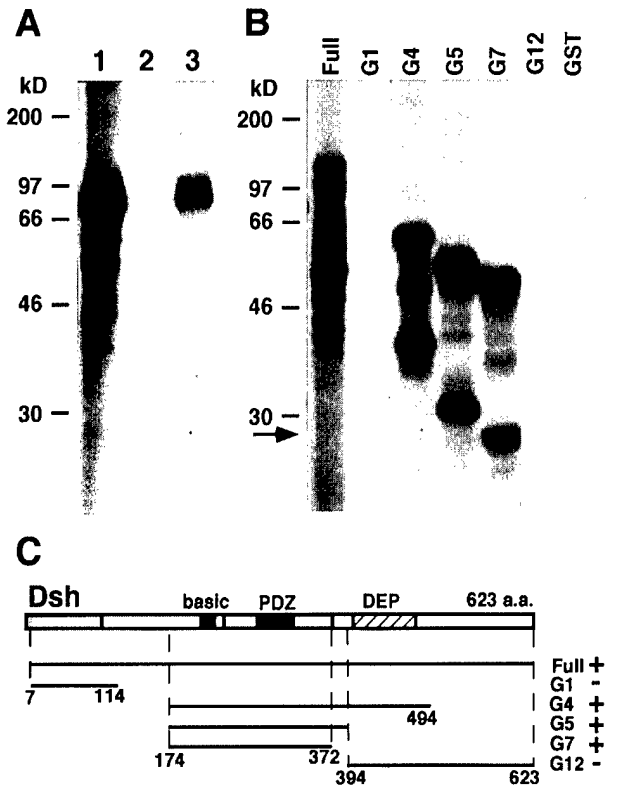

Fig. 2. Dsh kinase is associated with Dsh and phosphorylates GST–Dsh *in vitro*. (**A**) The Dsh-associated kinase can be disrupted from Dsh by an increase in ionic strength and can be reconstituted on Dsh. Shown are *in vitro* kinase reactions on Dsh immunoprecipitations from Clone 8 *dsh-myc* cells induced for Dsh overexpression. Prior to the *in vitro* kinase reaction the Dsh immunoprecipitations were treated as follows. Lane 1: wash with physiological salt concentrations (150 mM NaCl). Lane 2: wash with high salt concentrations (1 M NaCl). Lane 3: wash with high salt concentrations (1 M NaCl) and then incubation with the high salt wash of a Dsh immune complex that was diluted to 150 mM NaCl. (**B**) The central domain of Dsh associates with the kinase and is phosphorylated by the kinase. The high salt wash that removes the kinase activity from Dsh (panel A, lane 2) was diluted to 150 mM NaCl and added to GST–Dsh fusion proteins bound to glutathione–Sepharose beads. After incubation to allow association between kinase and GST–Dsh, the beads were washed and used in an *in vitro* kinase reaction; the products of the kinase reactions were resolved by SDS–PAGE. The slowly migrating bands represent full-length proteins while the lower bands are breakdown products. The arrow indicates the position of GST alone. (**C**) Summary of the data in (B).

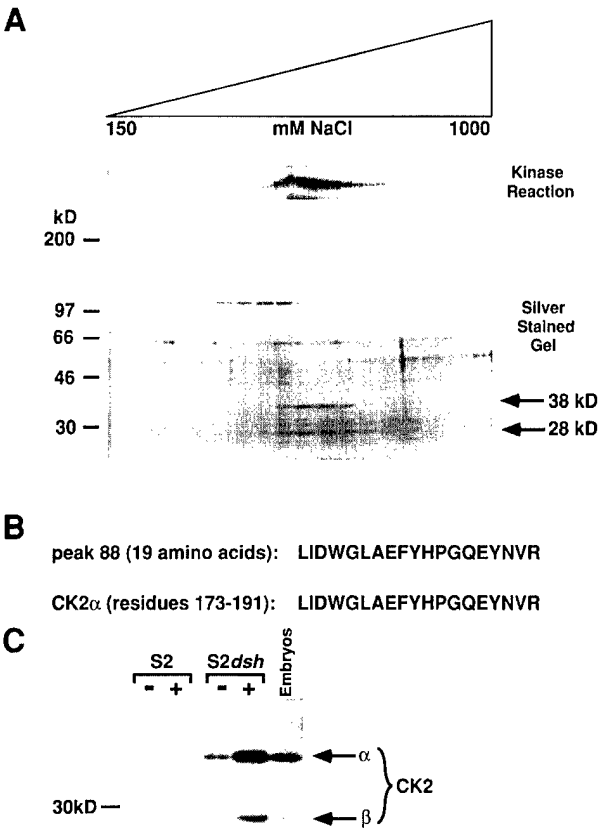

Fig. 3. Dsh kinase is CK2. (**A**) Purification of Dsh kinase. A total cell lysate from S2*dsh* cells was passed over an anti-Dsh antibody affinity column. After extensive washing, a linear NaCl gradient (150 mM to 1 M) was applied, individual fractions were assayed for their kinase activity on a GST–Dsh (G6), and products were resolved on an SDS–polyacrylamide gel that was subsequently silver-stained. The arrows mark the position of the bands co-purifying with the kinase activity. (**B**) The 38 kDa protein co-purifying with the Dsh Kinase activity is CK2α. The 38 kDa protein band co-purifying with the Dsh kinase activity was excised from the gel and digested with trypsin. Individual peptides were purified and one peptide (peak 88) was sequenced. The peptide sequence of this peptide is shown and lined up with the corresponding sequence of CK2α. (**C**) CK2 is associated with overexpressed Dsh. Dsh protein was immunoprecipitated from equal amounts of total protein of S2, S2*dsh* cells and embryos, and the co-immunoprecipitating proteins were immunoblotted with anti-CK2 antibody. This antibody recognizes both the α and β subunits of CK2. Cells were either treated (+) or not treated (–) with 0.5 mM $CuSO_4$ to modulate expression of the transfected *dsh* gene.

which includes the basic domain and the PDZ domain, contains the region required for the association with the Dsh-specific kinase and most, if not all, of the phosphorylation sites on Dsh (summarized in Figure 2C).

Identification of the Dsh-associated kinase as CK2

We used the specific binding of the kinase to Dsh as a first step in the purification of this enzyme. A Triton X-100 soluble protein extract from S2*dsh* cells was applied to an anti-Dsh antibody affinity column; the column was then extensively washed with lysis buffer prior to the application of a linear salt gradient. Individual fractions from this column were assayed for kinase activity towards a GST–Dsh fusion protein (G6, a more highly expressed variant of G5 with an additional 22 amino acids at the amino-terminus). At a salt concentration of 400 mM, a Dsh kinase activity eluted from the affinity column (Figure 3A, middle panel). Fractions were also separated by SDS–PAGE, and the gel was silver-stained. The kinase active fractions contained two bands with apparent molecular weight of 38 and 28 kDa (Figure 3A, bottom panel).

Kinase active fractions were combined and re-fractionated on an anion exchange column. Fractions containing the kinase activity again contained the 38 and 28 kDa proteins (data not shown), suggesting that these two proteins comprised the Dsh-associated kinase. Very few other proteins were visible in the kinase active fractions on a silver-stained gel (data not shown), implying that the material from the anti-Dsh affinity and anion exchange column was relatively pure.

The predominant bands of 38 and 28 kDa were isolated and subjected to peptide sequencing. Briefly, ~1.5 μg of the 38 kDa protein was digested with trypsin, and tryptic

peptides were purified and subjected to automated peptide sequencing. One tryptic peptide (peak 88) was sequenced, and the amino acid sequence revealed a perfect match with the α subunit of *Drosophila melanogaster* CK2 (Figure 3B; Saxema *et al.*, 1987).

To confirm that the Dsh-associated kinase is indeed CK2, an antibody raised to the α and β subunits of *D.melanogaster* CK2 (kindly provided by C.V.C.Glover; described in Dahmus *et al.*, 1984) was used in Western blotting. Cell extracts from embryos, Clone 8 and S2 cells contained the α and β subunits of CK2 (data not shown). An anti-Dsh immunoprecipitate from S2 cells contained very low levels of CK2. In contrast, Dsh immunoprecipitates from lysates of S2*dsh* cells induced to overexpress the transfected *dsh* gene contained significantly higher levels of CK2 (Figure 3C). Furthermore, CK2 co-immunoprecipitated with Dsh in lysates prepared from an overnight collection of embryos (Figure 3C), demonstrating that CK2 is associated with Dsh in the *Drosophila* embryo.

Phosphoamino acid analysis of in vivo and in vitro phosphorylated Dsh

To determine the nature of the individual phosphoamino acids in Dsh, we performed a phosphoamino acid analysis. Consistent with CK2 being a serine/threonine kinase, phosphoamino acid analysis of hydrolyzed Dsh protein demonstrates that most of the phosphorylation occurs on serine, some on threonine, and none on tyrosine (Figure 4). *In vitro* labeled Dsh contains more phospho-threonine than *in vivo* labeled Dsh.

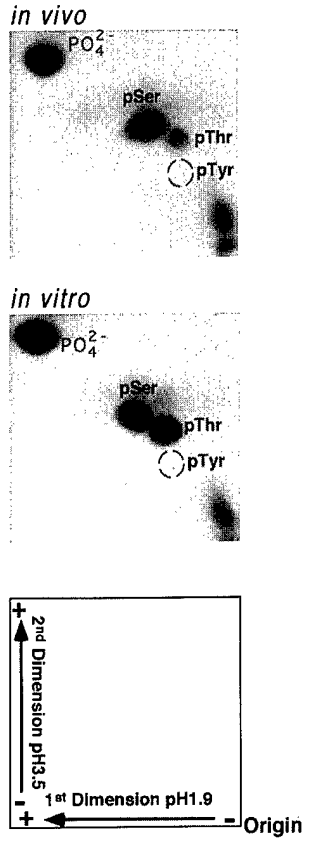

Fig. 4. Phosphoamino acid analysis of *in vivo* and *in vitro* labeled Dsh protein reveals that Dsh is phosphorylated on serine and threonine residues. Tryptic digestion products of *in vivo* and *in vitro* labeled Dsh were acid hydrolyzed to produce individual amino acids and then resolved in two dimensions on TLC cellulose plates. To mark the position of individual phosphoamino acids, 2 mg of each phosphoserine (pSer), phosphothreonine (pThr) and phosphotyrosine (pTyr) were included in the electrophoresis. The position of the marker phosphoamino acids was visualized by staining with ninhydrin. The diagram illustrates the direction and conditions of the two-dimensional electrophoresis.

Dsh is phosphorylated on similar sites in vitro and in vivo

In vitro, kinases are known to phosphorylate several substrates promiscuously. We used two dimensional maps of tryptic phosphopeptide fragments of the substrate to assess whether similar sites on Dsh are phosphorylated *in vivo* as *in vitro*, as a first estimate of whether the same enzyme phosphorylates Dsh *in vivo* and *in vitro*.

As shown in Figure 5, the tryptic maps of *in vitro* and *in vivo* phosphorylated Dsh are very similar, with three reproducible phosphotryptic products (spots A, B and C) common between *in vivo* and *in vitro* labeled Dsh. When tryptic peptides from *in vivo* and *in vitro* labeled Dsh are mixed and resolved on TLC cellulose plates, the same number of spots (A, B and C) are observed. This demonstrates that identical tryptic peptides are phosphorylated *in vivo* as *in vitro* and suggests that similar sites are being phosphorylated. Furthermore, *in vivo* labeled Dsh from S2*dsh* and S2*Dfz2* overexpressing cells (see below) both produced similar tryptic phosphopeptide maps (data not shown), suggesting that the same kinase is responsible for the phosphorylation of Dsh in these two cell lines.

CK2 is associated with Dsh in cells overexpressing Dfz2

Recently, it has been demonstrated that *Dfz2* protein acts as a receptor for Wg (Bhanot *et al.*, 1996). To determine whether overexpression of *Dfz2* has an effect on the phosphorylation state of the Dsh protein and on the Dsh–CK2 complex, S2 cells were transfected with a *Dfz2* cDNA under the control of the metallothionein promoter (S2*Dfz2*, described in Bhanot *et al.*, 1996). The *Dfz2* transgene can be induced to high levels by addition of Cu^{2+} to the medium.

In parental S2 cells, which do not express Dfz2 protein (Bhanot *et al.*, 1996), Dsh migrates as a single band of 70 kDa (Figures 1A and 6, first panel). Uninduced S2*Dfz2* cells contain low, but detectable, levels of Dfz2 protein and display a series of Dsh bands that migrate more slowly than Dsh in S2 cells. Addition of Cu^{2+} to the medium induced high levels of Dfz2 protein (Figure 6, second panel) and converted the series of Dsh bands to one main band of protein with an apparent molecular weight of 80 kDa. Treatment of cell lysates of S2*Dfz2* cells with potato acid phosphatase converts the slowly migrating Dsh bands to a single band with the same mobility as the Dsh protein in S2 cells (data not shown), demonstrating that the increase in molecular weight is the result of phosphorylation. In contrast to S2 cells, Clone 8 cells express *Dfz2*, and Dsh migrates not as a single band but as a series of differently phosphorylated forms (data not shown). This observation further supports our finding that *Dfz2* expression regulates the phosphorylation state of Dsh. *Dfz-2* overexpression had no effect on the expres-

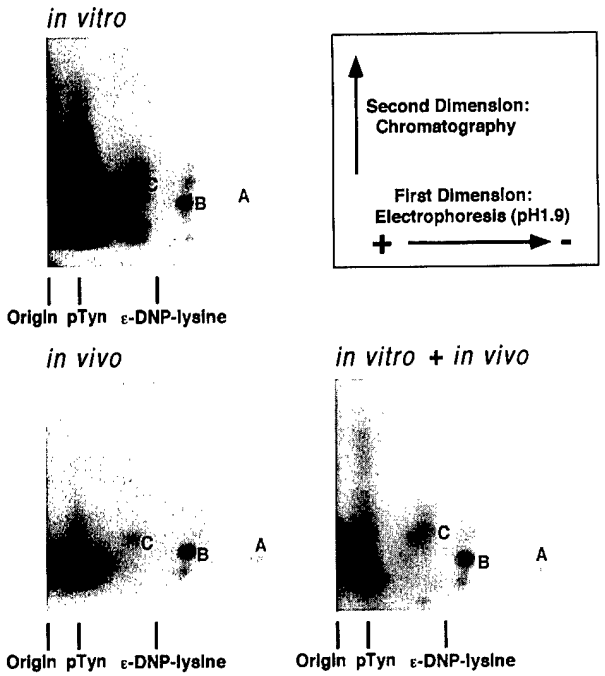

Fig. 5. Phosphotryptic maps of Dsh labeled *in vivo* and *in vitro* are identical. *In vivo* and *in vitro* labeled Dsh protein was resolved by SDS–PAGE and transferred to nitrocellulose. The Dsh bands were then digested with trypsin and proteolytic fragments were resolved in two dimensions on TLC cellulose plates. The diagram represents the direction of electrophoresis and chromatography. ε-DNP-lysine and phosphotyramine (p-Tyn) were used as markers.

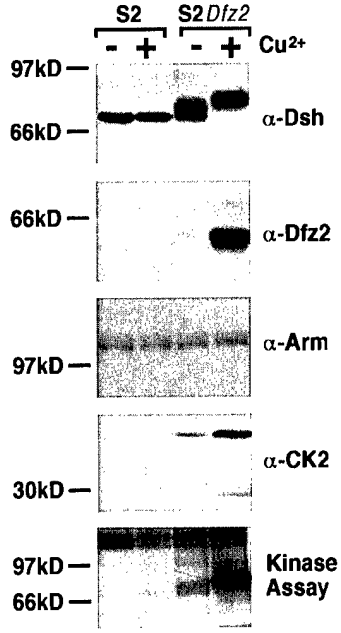

Fig. 6. CK2 is associated with phosphorylated Dsh in cells overexpressing *Dfz2*. S2 and S2*Dfz2* cells were either treated (+) or not treated (–) with $CuSO_4$ to modulate expression of the transfected genes. Equal amounts of total cell lysates were immunoblotted with anti-Dsh antibody (first panel), anti-Dfz2 antibody (second panel) or anti-Arm antibody (third panel). Dsh immunoprecipitates from equal amounts of protein of S2 and S2*Dfz2* cell lysates were immunoblotted with anti-CK2 antibody, which recognizes both the 38 kDa α-subunit and the 28 kDa β-subunit of CK2 (fourth panel), or incubated with [γ-^{32}P]ATP for an *in vitro* kinase reaction (fifth panel).

sion of *dsh* as determined by Northern blotting (data not shown).

Unlike overexpression of *dsh*, which leads to the accumulation of Arm protein (Figure 1A), overexpression of *Dfz2* does not (Figure 6, third panel). Thus, the phosphorylation of Dsh stimulated by *Dfz2* overexpression is not sufficient for the transduction of the signal to Arm. Overexpression of *Dfz2* also leads to an increase in the amount of CK2 associated with Dsh (Figure 6, fourth panel) and to an increase in kinase activity associated with immunoprecipitated Dsh protein (Figure 6, fifth panel). These results demonstrate that CK2 associates with endogenous Dsh protein when the phosphorylation of Dsh protein is elevated by the overexpression of Dfz2, a receptor for the Wingless protein.

Discussion

In this paper we show that CK2 associates with Dishevelled (Dsh) and phosphorylates Dsh *in vitro*. This association between CK2 and Dsh occurs in the embryo and is greatly promoted when *dsh* or *Dfz2*, which encodes a receptor for Wingless (Wg), are overexpressed. Furthermore, phosphoamino acid analysis and tryptic phosphopeptide maps suggest that CK2 phosphorylates Dsh *in vivo*.

CK2 is a highly conserved protein kinase with homologs found in every tested organism from yeast to humans. In yeast, the CK2α gene is essential for viability (Bidwai *et al.*, 1992), and the high degree of conservation suggests an essential role in all organisms. No mutations have been identified for *Drosophila* CK2α and β (Saxema *et al.*, 1987) which map to the chromosomal locations 80A and 10E, respectively (Lindsley and Zimm, 1992).

CK2 has been studied extensively at the biochemical level but very little is known about its functions *in vivo* (reviewed in Issinger, 1993; Allende and Allende, 1995). The kinase activity of CK2 appears to be constitutively active, and no significant regulation by external stimuli has been reported. Our finding that expression of a receptor for Wingless, *Dfz2*, controls the association of CK2 with Dsh and the phosphorylation of Dsh, provides one of the first examples of regulation of CK2 association with a substrate. A vast number of *in vivo* substrates have been identified for CK2 (reviewed in Marshak and Carroll, 1991), and, as is the case with Dsh, the function of these phosphorylation events has not been elucidated. It has, however, been demonstrated that phosphorylation by CK2 leads to the breakdown of IκB (McElhinny *et al.*, 1996).

The optimal motif for CK2 phosphorylation is a serine or threonine residue in an acidic context with a minimal requirement of one acidic residue at the +3 position (Marshak and Carroll, 1991; Rihs *et al.*, 1991; Songyang *et al.*, 1996). There are two serines and two threonines that fit this consensus within the minimal Dsh fragment (G5, see Figure 2) that is efficiently phosphorylated by CK2 *in vitro*. Site-directed mutagenesis of these sites is in progress.

We show here that CK2 associates with Dsh and phosphorylates it, but we have not determined which of these events occurs first in the cell. Unphosphorylated Dsh clearly has some affinity for CK2 since a bacterially produced GST–Dsh fusion protein (which is most likely

not phosphorylated to a significant level) can associate with CK2 *in vitro*. However, *in vivo* phosphorylated Dsh (either by *dsh* or *Dfz2* overexpression) is associated with more CK2 than is underphosphorylated Dsh. This suggests a model in which CK2 can bind to underphosphorylated Dsh with low affinity and phosphorylate it. The phosphorylated Dsh then has a higher affinity for CK2 leading to an increase in the amount of Dsh–CK2 complex. This type of interaction may be similar to the interaction of an SH2 or PTB domain with a phosphotyrosine (reviewed in Pawson, 1995) or the interaction between 14-3-3 proteins and phosphoserine (Muslin *et al.*, 1996). Whether phosphorylation of Dsh increases the binding of CK2 to Dsh still remains to be shown. Phosphorylation of Dsh may also induce a conformational change that results in a stronger association between Dsh and CK2. Alternatively, overexpression of *dsh* or *Dfz2* may promote the association of CK2 with Dsh, leading to an increase in Dsh phosphorylation.

In a recent paper, we demonstrated that Wingless signaling leads to the increased phosphorylation of Dsh, and that this phosphorylation of Dsh correlated with the accumulation of the Arm protein (Yanagawa *et al.*, 1995). However, we now find that overexpression of a receptor for Wg, *Dfz2*, leads to the phosphorylation of Dsh but fails to induce elevated levels of Arm protein. Thus, Dsh phosphorylation alone is not sufficient for the transduction of the Wg signal to Arm. It remains possible, however, that the phosphorylation of Dsh by CK2 and by perhaps other unidentified kinases is required but not sufficient for the transduction of the Wg signal. In this model, Dsh is phosphorylated on multiple sites, some of which are regulated by CK2. Wg signaling would lead to yet other, presently unidentified, changes in Dsh that allow the signal to be transduced to downstream effectors. In this respect, it is noteworthy that the two Wg responsive cell lines, Clone 8 and S2 cells overexpressing *Dfz2*, contain phosphorylated Dsh, while the Wg non-responsive S2 cells contain unphosphorylated Dsh. Alternatively, the phosphorylation of Dsh is not required for the transduction of a Wnt signal but is only a consequence of the signal. Such phosphorylation events may be involved in down-regulating the Wg signal.

Clearly, the function of the phosphorylation of Dsh by CK2 is not yet established, and further experiments, such as site-directed mutagenesis of potential phosphorylation sites and assessment of the activity of such mutant alleles in embryos, are necessary.

Materials and methods

Cell culture

Schneider 2 (S2) cells and S2 cells overexpressing *dsh* or *Dfz2* under the control of the metallothionein promoter (S2*dsh* and S2*Dfz2*, respectively) were grown in Schneider's *Drosophila* medium (Gibco BRL) supplemented with 12.5% fetal bovine serum (Sigma), 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco BRL) at 25°C. The wing imaginal disc cell line, Clone 8 (Cl8), was grown in Shields and Sang M3 insect medium (Sigma) supplemented with 2% FBS, 2.5% fly extract, 5 μg/ml insulin and penicillin and streptomycin. S2*dsh*, Cl8*Dsh-myc* and Cl8Δ*C-term Dsh-myc* cells are described elsewhere (Yanagawa *et al.*, 1995; ΔC-term Dsh-myc is refered to as D1). The generation of the cell line S2*Dfz2* is described in Bhanot *et al.* (1996). These cells were induced to overexpress the transfected genes by adding 0.5 mM $CuSO_4$ for 4–16 h.

Preparation of cell lysates

For the purification of CK2/Dsh-associated kinase ~2.5×10^{10} S2*dsh* cells (6 l of dense culture medium) were pelleted at 1000 *g* and washed three times with cold phosphate buffered saline (PBS). The cell pellet was then resuspended in 130 ml ice-cold lysis buffer (1% Triton X-100, 50 mM Tris–HCl pH 8.0, 150 mM NaCl) containing protease inhibitors [1 mM Pefabloc® SC (Boehringer Mannheim), 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin (Sigma)] and 0.2 μM phosphatase inhibitor microcystin-LR (Gibco BRL) and incubated on ice for 20 min. The cell lysate was pre-cleared by spinning at 1000 *g* for 10 min at 4°C. The supernatant was centrifuged at 100 000 *g* for 1 h at 4°C. The protein concentration was determined by the method of Bradford (Bio-Rad Protein Assay). The lysate from an overnight collection of embryos was obtained by douncing on ice dechorionated embryos in lysis buffer containing protease inhibitors in a tight fitting dounce. The lysate was then centrifuged at 20 800 *g* for 10 min to remove insoluble cell debris.

Generation of GST–Dsh fusion proteins

The fusion constructs between GST and fragments of Dsh, Full, G4, G5 and G7 were constructed from pBluescriptIIKS$^+$*dsh* by S.Yanagawa as follows:

Full (amino acids 7–623): PCR using the primers:

5′ primer: TAGGATCCGGCGGGCAGGAGACGAAGGTGT.
3′ primer: CCGAATTCCAATACGTAATTAGATACGGA.

The PCR product was digested with *Bam*HI and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites of pGEX-2TK.

G7 (amino acids 174–372): PCR using the primers:

5′ primer: TAGGATCCCCGCCACCGCTCACATATCAA.
3′ primer: CCGAATTCTGTGAGGGCCTGAGTGTGCGC.

The PCR product was cloned into pGEX-2TK as for Full.

G4 (amino acids 174–494): PCR using the primers:

5′ primer: same as for G7.
3′ primer: same as for Full.

The PCR product was digested with *Bam*HI and *Pvu*II and cloned into the *Bam*HI and *Sma*I sites of pGEX-2TK.

G5 (amino acids 174–394): The PCR product used for G4 was digested with *Xho*I, blunted, digested with *Bam*HI and cloned into *Bam*HI and *Sma*I sites of pGEX-2TK.

G6 (amino acids 152–394): pBluescriptIIKS$^+$*dshmyc* was digested with *Pvu*II and *Xho*I to generate a 727 bp fragment which was cloned into the *Sma*I and *Xho*I sites of pGEX4T-2.

G12 (amino acids 394–623): pBluescriptIIKS$^+$*dsh* was digested with *Xho*I and *Not*I to generate a 1216 bp fragment which was cloned into the *Xho*I and *Not*I sites of pGEX4T-1.

GST fusion proteins were produced and purified as described by the vendor Pharmacia.

Antibodies

For immunoprecipitation of Dsh, a rabbit antibody (anti-Dsh) raised to the carboxy-terminus was obtained (see below). For immunoblotting, either anti-Dsh or a rat anti-Dsh antibody raised to the amino-terminus of Dsh (Yanagawa *et al.*, 1995) was used. Dsh-myc or ΔC-term Dsh-myc was immunoprecipitated with anti-myc antibody (9E10). Dfz2 was detected by immunoblotting with an anti-Dfz2 antibody raised to the amino-terminus of Dfz2 (Bhanot *et al.*, 1996). CK2 and Arm were detected with anti-CK2 (Dahmus *et al.*, 1984) and anti-Arm (7A1, Peifer, 1993) antibodies, respectively.

Preparation of anti-Dsh affinity column

The anti-Dsh antiserum, anti-Dsh, was obtained by immunizing rabbits with the bacterially produced fusion protein G12. The antiserum was affinity purified against the GST fusion protein G12. Affinity purified anti-Dsh antibody was covalently cross-linked to protein A–Sepharose with Dimethylpimelimidate using the ImmunoPure® protein A–IgG Orientation Kit (Pierce). The resulting protein A–Sepharose/anti-Dsh matrix was then used to pour a column (total bed volume ~5 ml) which was used for affinity purification of Dsh and its associated kinase.

Affinity purification of Dsh kinase

Lysis of 2.5×10^{10} cells yielded ~3 g of Triton X-100 soluble protein as determined by the method of Bradford (Bio-Rad Protein Assay). The

following steps were all carried out at 4°C. 1.5 g of the lysate was preincubated with Sepharose CL-4B (Sigma), and then applied to the anti-Dsh affinity column at a flow rate of 0.075 ml/min using the LKB Pump P-1 (Pharmacia). The column was washed extensively with lysis buffer (1% Triton X-100, 50 mM Tris–HCl, pH 8, 150 mM NaCl) at a flow rate of 1 ml/min. A 15 ml 150–1000 mM NaCl gradient in 1% Triton X-100 and 50 mM Tris–HCl, pH 8 was applied onto the column at a flow rate of 0.5 ml/min. 0.5 ml fractions were collected and later assayed for kinase activity and for protein content by silver-stained gels. After this first run, the column was stripped with 6.4 M urea, 1 M NaCl and regenerated by washing with lysis buffer. The second half of the lysate (1.5 g) was applied, washed and eluted as above.

Anion exchange chromatography of Dsh kinase

The kinase active fractions from the two runs of the anti-Dsh affinity column were pooled and the NaCl concentration was diluted to 150 mM. The pooled fractions were applied onto a HiTrap^{TM}Q (Pharmacia) anion exchange column at a flow rate of 0.5 ml/min. The column was washed with 10 ml lysis buffer, and then with 20 ml 20 mM Triethanolamine, pH 7.3, 150 mM NaCl. A 15 ml 150 mM to 1000 mM NaCl gradient in 20 mM Triethanolamine, pH 7.3 was applied onto the column at a flow rate of 0.5 ml/min. 0.5 ml fractions were collected and later assayed for kinase activity (see below) and for protein content by silver-stained gels (as described in Giulian *et al.*, 1983).

TCA precipitation and gel electrophoresis of Dsh kinase

The kinase active fractions from the anion exchange column were pooled and precipitated by adding an equal volume of 24% trichloroacetic acid (TCA) and incubating on ice for 30 min. The precipitate was pelleted, washed with acetone, air dried, resuspended in 40 µl protein loading dye (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.73 M β-mercaptoethanol), boiled and run on an SDS 12.5% polyacrylamide gel. The gel was stained with 0.1% Coomassie, 40% methanol, 10% acetic acid for 30 min and then destained for 4 h with several changes of 50% methanol, 10% acetic acid. The bands of 38 and 28 kDa were cut out, frozen and sent to the Keck Foundation at Yale University for protein digestion and sequencing. Briefly, ~1.5 µg of the 38 kDa protein was eluted from the gel and digested with trypsin. Tryptic peptides were purified and subjected to automated peptide sequencing.

Kinase assays

For the *in vitro* kinase reactions in Figures 1, 2A and 6, the anti-Dsh immune complex was washed four times with lysis buffer and twice with distilled water and then incubated with 30 µl kinase buffer (20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 µM ATP, 1 mM DTT and 7 µCi [γ-^{32}P]ATP) for 1 h at room temperature.

In Figure 2B, the eluted kinase activity was incubated with various purified GST fusion proteins immobilized on glutathione–Sepharose 4B (Pharmacia). The kinase–GST–Dsh–Sepharose complexes were washed four times with lysis buffer, twice with distilled water and then incubated with kinase buffer as above. Kinase reactions were stopped by adding protein gel loading dye and boiling for 5 min. The products of the kinase reactions were resolved by SDS–PAGE.

To assay for kinase activity in the fractions from the anti-Dsh affinity column and from the anion exchange column, 1 µl (1/500th) of each fraction was incubated with 10 µl Sepharose–G6 beads (a 1:1 slurry containing ~1 µg of fusion protein) in 0.5 ml lysis buffer for 1 h at 4°C to allow for association between the kinase and its substrate. The Sepharose–G6 beads were washed with 1 ml distilled water. 20 µl of kinase buffer was added to the Sepharose–G6 pellet and incubated at 25°C for 1 h. Protein gel loading dye was added, samples were boiled and resolved by SDS–PAGE. The gel was stained and fixed in 0.002% Coomassie, 20% methanol, 7% acetic acid for 30 min and then dried. Labeled protein bands were visualized by autoradiography.

Immunoblotting and immunoprecipitations

Triton X-100 soluble proteins were resolved by SDS–PAGE and electroblotted to nitrocellulose filter (Schleicher and Schuell) in 25 mM Trizma, 0.192 M glycine and 20% methanol for 1.5 h in the cold room at 500 mA. Filters were blocked in 3% non-fat dry milk (Carnation), 1% bovine serum albumin (BSA) in Tris buffered saline (TBS: 20 mM Tris–HCl pH 8, 150 mM NaCl) and 0.2% Tween-20 (TBST). Rat anti-Dsh, affinity purified anti-Dsh, anti-CK2 (kindly provided by C.V.C.Glover), anti-Arm and anti-Dfz2 were added to the filters at dilutions of 1:2000, 1:2000, 1:6000, 1:1000 and 1:5000, respectively, and incubated overnight at 4°C. The filters were washed in TBST, incubated with secondary antibody conjugated to horse radish peroxidase (HRP, Bio-Rad). After washing the filters, the chemiluminescent reagent (ECL, Amersham) was added.

For immunoprecipitations, 5 µl of anti-Dsh, 20 µl anti-Dsh coupled to protein A–Sepharose (see above under 'Preparation of anti-Dsh affinity column'), or 20 µl anti-Myc (9E10) were added to 1–5 mg total cell lysate and incubated overnight at 4°C. To precipitate the antibodies, 20 µl of protein A–Sepharose (PAS, 1:1 slurry) was added and incubated for 1 h at 4°C. The immune complexes were washed four times with lysis buffer and then boiled in sample dye prior to SDS–PAGE. Western blotting of the Dsh immunoprecipitate with anti-CK2 antibody (Figure 4) produced high levels of background signal due to the reactivity of the secondary goat anti-rabbit antibody with the rabbit anti-Dsh antibody chains on the nitrocellulose blot. To circumvent this problem, the Dsh immune complex was first washed extensively with lysis buffer containing a physiological NaCl concentration (150 mM). Associated proteins like CK2 were eluted from the immune complex by washing it five times with 150 µl lysis buffer containing 1 M NaCl; this high salt eluate was TCA precipitated by adding an equal volume of 24% TCA. Precipitated protein was boiled in sample dye prior to SDS–PAGE.

The amount of CK2 associated with phosphorylated Dsh in S2*Dfz2* cells was estimated as follows: the signal strength of an anti-CK2 immunoblot on 50 µg total cell extract was equivalent to the signal strength of a CK2 immunoblot on a Dsh immunoprecipitate from 16 mg total cell extract. Since all the Dsh was immunodepleted from the cell extract, we can calculate the percentage of CK2 associated with Dsh: $50/16000 \times 100 = 0.31\%$.

In vivo and in vitro labeled Dsh protein for phosphoamino acid analysis and tryptic phosphopeptide mapping

In vitro labeled Dsh protein was obtained as described under 'Kinase assays.' *In vivo* labeled Dsh was obtained as follows: a confluent T-75 flask of Cl8*dsh-myc* cells was starved in phosphate-free M3 medium (Kemp Biotechnologies, Inc.) in the presence of 0.5 mM $CuSO_4$ for 1 h. The medium was removed and replaced with 5 ml phosphate free M3 medium containing 0.5 mM $CuSO_4$ and 5 mCi [^{32}P]orthophosphate (Amersham) and incubated for 5 h to overnight at 25°C. The cells were washed and lysed as described above. The Dsh protein was immunoprecipitated from whole cell extracts with α-myc antibody (9E10). Protein gel loading dye was added to the washed immune complex prior to SDS–PAGE and electroblotting to nitrocellulose. Immobilized and labeled Dsh protein (*in vitro* and *in vivo*) was visualized by autoradiography and excised from the filter. Digestions of the Dsh protein, phosphoamino acid analysis and tryptic phosphopeptide mapping were performed as described (Boyle *et al.*, 1991; Luo *et al.*, 1991).

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Wnt signaling: a common theme in animal development

Ken M. Cadigan and Roel Nusse

Howard Hughes Medical Institute, Department of Developmental Biology, Beckman Center, Stanford University Medical Center, Stanford, California 94305-5323 USA

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REVIEW

Wnt signaling: a common theme in animal development

Ken M. Cadigan and Roel Nusse[1]

Howard Hughes Medical Institute, Department of Developmental Biology, Beckman Center, Stanford University Medical Center, Stanford, California 94305-5323 USA

Wnt proteins are now recognized as one of the major families of developmentally important signaling molecules, with mutations in *Wnt* genes displaying remarkable phenotypes in the mouse, *Caenorhabditis elegans*, and *Drosophila*. Among functions provided by Wnt proteins are such intriguing processes as embryonic induction, the generation of cell polarity, and the specification of cell fate. Until recently, our knowledge of the molecular mechanism of Wnt signaling was very limited, but over the past year, several major gaps have been filled. These include the identification of cell-surface receptors and a novel mechanism of relaying the signal to the cell nucleus. In addition, several components of Wnt signaling are implicated in the genesis of human cancer. These insights have come from different corners of the animal kingdom and have converged on a common pathway. At this junction in this rapidly evolving field, we review our current understanding of Wnt function and signaling mechanisms, doing so in a comparative approach. We have put emphasis on the latest findings, highlighting novelty and underscoring questions that remain. For additional literature, we refer to several previous reviews (McMahon 1992; Nusse and Varmus 1992; Klingensmith and Nusse 1994; Miller and Moon 1996; Moon et al. 1997). We have limited the number of references, particularly in the tables. Fully referenced forms of these tables can be found on the Wnt homepage (http://www-leland.stanford.edu/~rnusse/wntwindow.html).

The Wnt Family

Wnt genes are defined by sequence homology to the original members *Wnt*-1 in the mouse (first called *int*-1; Nusse and Varmus 1982; Van Ooyen and Nusse 1984) and *wingless* (*wg*) in *Drosophila* (Cabrera et al. 1987; Rijsewijk et al. 1987). They encode secreted glycoproteins, usually 350–400 amino acids in length. Homologous genes have been found in increasing numbers in organisms ranging from mammals to the nematode *C. elegans*. The degree of sequence identity in Wnt proteins is minimally 18%, including a conserved pattern of 23–24 cysteine residues, in addition to other invariant amino acids. By phylogenetic analysis, several of these genes have been assigned as orthologs of each other across species, mostly within vertebrates (Sidow 1992). Vertebrate genomes also contain several combinations of highly similar *Wnt* genes, usually referred to as A-B pairs. Table 1 lists the known *Wnt* genes in the most relevant species as groups of orthologs.

wg in *Drosophila* (Sharma and Chopra 1976; Baker 1987; Rijsewijk et al. 1987) is the best understood *Wnt* family member and has been a paradigm in elucidating *Wnt* gene function in other organisms. The first insight into understanding the mechanism of Wnt signal transduction came from the existence of several fly genes with mutant phenotypes consistent with defects in Wg signaling (Nüsslein-Volhard and Wieschaus 1980; Wieschaus and Riggleman 1987; Perrimon et al. 1989). *porcupine* (*porc*), *dishevelled* (*dsh*), *armadillo* (*arm*; the *Drosophila* homolog of β-catenin) and *pangolin* (*pan*,DTcf) mutant embryos have segment polarity defects similar to *wg*, while *zeste-white 3* (*zw3*) mutants have the opposite phenotype. These genes have been ordered in a genetic pathway (Fig. 1; Klingensmith et al. 1994; Noordermeer et al. 1994; Peifer et al. 1994b; Siegfried et al. 1994; Brunner et al. 1997; Van de Wetering et al. 1997), and recent studies have focused on understanding the biochemical relationship between the protein products. This effort has been greatly augmented by the study of the vertebrate counterparts of these genes, giving us our current understanding of *Wnt* signal transduction summarized in this review.

More *Wnt* phenotypes

Many of the known *Wnt* loss-of-function mutations have been generated in the mouse, and some highly intriguing phenotypes are seen. For example, inactivation of *Wnt*-7A results in animals with ventralized limbs (Parr and McMahon 1995). This phenotype concurs with the dorsal epidermal expression pattern of *Wnt*-7A and with the consequences of ectopic expression of the gene in chicken limb buds, which dorsalizes the limbs (Yang and Niswander 1995). Similarly interesting is the phenotype of *Wnt*-4 mutations: the absence of kidneys (Stark et al. 1994). This role of *Wnt*-4 in kidney development is underscored by ectopic expression studies, showing that

[1]Corresponding author.
E-MAIL rnusse@cmgm.stanford.edu; FAX (650) 723-1399.

Table 1. Wnt *genes in various organisms*

Gene	Mouse	Human	*Xenopus*	Chicken	Zebrafish	*Drosophila*	*C. elegans*[a,b]
Wnt-1	●	●	●		●	*wg*	*Ce-Wnt*-1
Wnt-2	●	●	●		●		*Ce-Wnt*-2
Wnt-2B		●	●				*lin*-44
Wnt-3	●	●	●		●		*mom*-2
Wnt-3A	●		●				*egl*-20
Wnt-4	●	●	●	●	●		
Wnt-5A	●	●	●	●	●	*DWnt*-3/5	
Wnt-5B	●						
Wnt-6	●		●				
Wnt-7A	●	●	●	●	●	*DWnt*-2	
Wnt-7B	●	●	●				
Wnt-7C			●				
Wnt-8A	●		●	●	●		
Wnt-8B[c]	●	●	●		●		
Wnt-8C				●[d]			
Wnt-9[e]							
Wnt-10A	●		●		●		
Wnt-10B	●	●					
Wnt-11	●	●	●	●			
(*Wnt*-12, *Wnt*-13)[f]							
						DWnt-4[g]	

●Identification of the gene.
[a]The *C. elegans Wnt* genes are not assigned as orthologs of vertebrate genes.
[b]C. Kenyon (pers. comm.).
[c]Mouse *Wnt*-8B unpublished, isolated by John Mason (pers. comm.).
[d]Chicken *Wnt*-8C might be considered the true ortholog of mouse and *Xenopus Wnt*-8A, as these genes are very similar. In addition, there are no other chicken *Wnt*-8 genes yet, nor have separate orthologs of *CWnt*-8C been cloned from the mouse and the human.
[e]A partial sequence of *Wnt*-9 has been isolated from hagfisch and thresher shark only.
[f]There have been reports on *Wnt* genes called *Wnt*-12 and *Wnt*-13, but they are either identical to one another (*Wnt*-12 is the same as *Wnt*-10B) or similar (*Wnt*-13 should be called *Wnt*-2B). More information on the nomenclature and classification of *Wnt* genes can be found on the *Wnt* gene homepage (http://www-leland.stanford.edu/~rnusse/wntwindow.html).
[g]*DWnt*-4 is too divergent to be assigned as an ortholog.

this gene may function in the mesenchymal–epithelial transitions occurring during the formation of this organ (Herzlinger et al. 1994; Stark et al. 1994). (See Table 2 for a comprehensive list of *Wnt* mutations and phenotypes.)

Wnt *mutations in* C. elegans

The exciting recent findings on *Wnt* mutations in the nematode *C. elegans* have given the field another model system that rivals *Drosophila* in its power of genetic analysis. There are at least five *Wnt* genes in the worm, one of which (*mom-2*) is implicated in setting up the polarity of the embryo. In four-cell-stage embryos, the P2 cell, itself part of the germ-line lineage, polarizes the adjacent EMS cell which will then divide into a endodermal (E) and mesodermal (MS) precursor (for review, see Bowerman 1997; Figure 2). Genetic screens have identified a set of maternal genes called *mom* (for more mesoderm), where the E cell adopts a MS cell fate. One of these genes, *mom-2*, encodes a *Wnt* gene that is required in the P2 cell, suggesting that *mom*-2 is a major signal for the polarization of the EMS cell (Rocheleau et al. 1997; Thorpe et al. 1997). Other *mom* mutants include *mom*-1, encoding a homolog of *Drosophila porc* and *mom*-5, which belongs to the Frizzled (Fz) family of cell-surface proteins, recently implicated as Wnt receptors (Bhanot et al. 1996; Rocheleau et al. 1997). In addition, RNA interference experiments provide evidence for an Arm/β-catenin homolog functioning in this pathway (Rocheleau et al. 1997). The *pop*-1 gene (Lin et al. 1995), which has the opposite phenotype of the *mom* genes (transforming MS into an E cell fate) encodes a high mobility group (HMG) box transcription factor with homology to LEF-1 and the Tcf family, which interact with Arm/β-catenin to regulate Wnt targets in flies and vertebrates (this paper; for review, see Nusse 1997). The other identified *Wnt* mutation *lin*-44 (Herman et al. 1995) is also required for certain asymmetric cell divisions, in this case in the larval male tail, where *lin*-44 acts nonautonomously to polarize adjacent cells. These target cells require a Fz protein encoded by *lin*-17 for their asymmetric cell divisions to occur (Sawa et al. 1996). It appears therefore that the Wnt signaling pathway found in flies and vertebrates is similar in worms (see Fig. 3), though there may be important differences, which will be discussed.

Are Wnt *genes involved in embryonic axis specification in vertebrates?*

In *Xenopus*, injection of various *Wnt* genes as RNAs into

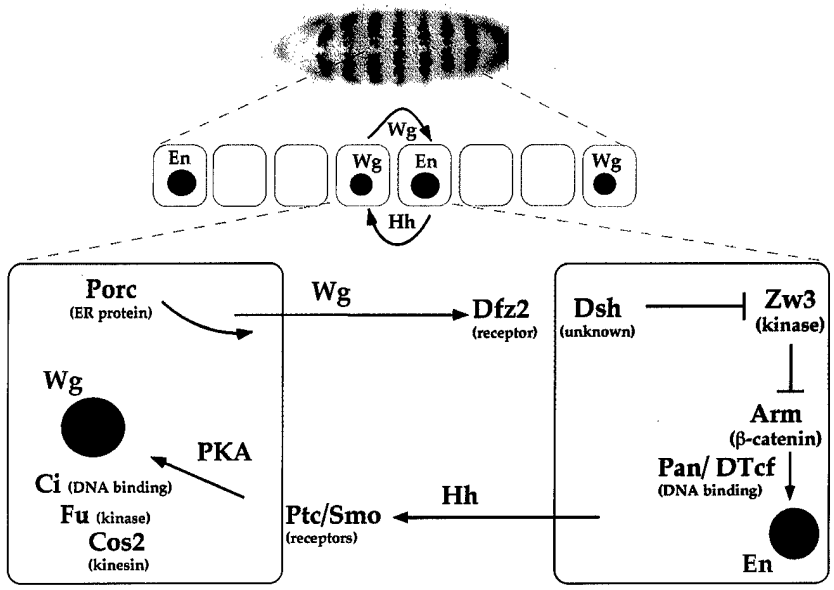

Figure 1. Intercellular signaling during *Drosophila* embryogenesis. (*Top*) A *Drosophila* embryo stained for expression of Wg (blue) and En (brown). Below is a representation of two parasegments (the parasegment boundary is between the Wg-and the En-expressing cells). Wg signals to maintain En expression; the En cells activate Wg expression by secreting the Hedgehog (Hh) protein. The Wg protein is secreted with the assistance of Porc, an ER transmembrane protein. Wg can act through the Dfrizzled-2 (Dfz2) receptor, although there is no genetic evidence that Dfz2 is required. Within the target cell, the PDZ-containing protein Dsh is required to transduce the signal leading to the inactivation of the protein kinase Zw3. In cells that do not receive Wg, Zw3 acts to destabilize the Arm protein. Together with DTcf (also known as *pan*) Arm can activate transcription of target genes, including *en*. The Hh protein, made by the En cells, binds to Patched (Ptc), which together with the Smoothened (Smo) protein forms a receptor complex. Within the target cell, the Hh signal is transduced by a complex between Cubitus interruptus (Ci), Fused (Fu), and Costal-2 (Cos2) to control Wg expression. Protein kinase A (PKA) probably acts in parallel to this pathway.

early ventral blastomeres leads to induction of dorsal mesoderm and a duplicated body axis (McMahon and Moon 1989; Moon 1993). Such *Wnt* genes can also rescue primary axis formation in developmentally compromised embryos. These observations are intriguing and have provided the field with useful assays for *Wnt* genes. Nonetheless, there are no data implicating an endogenous *Wnt* in induction of the primary axis, as no known *Wnt* is expressed in the right place at the right time. *XWnt*-8, for example, has potent axis-inducing effects (Smith and Harland 1991; Sokol et al. 1991) but is expressed too late, after the onset of zygotic transcription and in the wrong area [ventral marginal cells (Christian et al. 1991; Christian and Moon 1993)]. In addition, dominant-negative forms of Wnt (Hoppler et al. 1996), Fz (Leyns et al. 1997; Wang et al. 1997a), or Dsh (Sokol 1996) block secondary axis formation if coinjected with Wnt proteins, but they fail to block primary axis formation. At present, it seems unlikely therefore that a Fz–Wnt interaction is required for normal axis formation in frogs (Moon et al. 1997).

Table 2. Wnt *gene phenotypes in various organisms*

Gene	Organism	Phenotype
Wnt-1 (*swaying*)	mouse	deletion portion midbrain, cerebellum
Wnt-2	mouse	placental defects
Wnt-3A (*vestigial tail*)	mouse	tail, tailbud, caudal somites
Wnt-4	mouse	kidney defect
Wnt-7A	mouse	dorsal–ventral polarity limbs
wg	*Drosophila*	segment polarity; many others
DWnt-2	*Drosophila*	testis; adult muscles[a]
egl-20	*C. elegans*	Q-cell migration[b]
lin-44	*C. elegans*	T-cell polarity tail
mom-2	*C. elegans*	loss of endoderm, excess mesoderm in embryo

[a]K. Kozopas and R. Nusse (unpubl.).
[b]C. Kenyon (pers. comm.).

There is, however, compelling evidence that downstream members of the Wnt signaling pathway are essential for inducing the endogenous axis. Depletion of maternal β-catenin prevents the induction of the primary axis (Heasman et al. 1994). β-Catenin accumulates in the nuclei of dorsal blastomeres, consistent with activation of a Wnt pathway (Schneider et al. 1996; Larabell et al. 1997). This accumulation is blocked by overexpression of the zw3 homolog GSK-3 (Larabell et al. 1997). Likewise, overexpression of GSK-3 inhibits primary axis formation (Dominguez et al. 1995; He et al. 1995; Pierce and Kimelman 1996), as does a dominant-negative form of XTcf-3 that cannot bind β-catenin (Molenaar et al. 1996). Taken together, a picture emerges in which a non-Wnt mechanism inhibits GSK-3, stabilizing β-catenin and promoting a complex with XTcf-3 in dorsal nuclei (Fig. 3).

In the mouse, a naturally occurring recessive mutation, *fused*, has a duplicated axis phenotype similar to that seen after Wnt misexpression in *Xenopus* (Zeng et

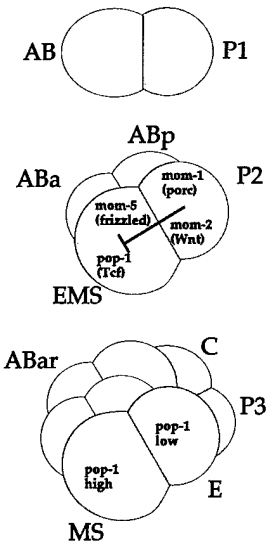

Figure 2. Intercellular signaling during *C. elegans* embryogenesis. The first division of the zygote gives rise to an anterior AB and a posterior P1 cell. The P1 cell divides into an anterior E/MS and a posterior P2 cell. A signal from P2 polarizes the E/MS blastomere, such that its anterior daughter (MS) will give rise to mesoderm and the posterior daughter (E) makes endoderm. In the absence of this signal, both daughters adopt the MS cell fate. The signal requires a Wnt (*mom*-2) and a *porc* homolog (*mom*-1), both required in P2. The *mom*-2 signal is probably received by the Fz homolog *mom*-5, resulting in down-regulation of the Tcf-related *pop*-1 protein in the E cell nucleus (compared to MS nuclei). The ABar blastomere, whose mitotic spindle orientation is disrupted in *mom*-1, *mom*-2, and *mom*-5 mutants, is also shown.

al. 1997). The cloned product of *fused*, a protein called Axin, can inhibit the formation of the primary axis in *Xenopus* when injected into dorsal blastomeres (Zeng et al. 1997). A ventrally injected dominant-negative version of the Axin protein results in frog embryos with defects similar to mouse *fused* mutants. In *Xenopus*, it appears that Axin inhibits β-catenin by activating GSK-3 or by acting on an unidentified protein between GSK-3 and β-catenin. The gastrulation phenotype of mice mutant for β-catenin (Haegel et al. 1995) is also consistent with an antagonistic relationship between Axin and β-catenin. Axin may act directly in the *Wnt* pathway, or it may be the target of the putative non-Wnt signal discussed above (Fig. 3).

Early misexpression of *Wnt*-8 (using the chicken gene called *Wnt*-8C (Hume and Dodd 1993) in mouse embryos can also induce a secondary axis (Pöpperl et al. 1997). As in frogs, endogenous mouse *Wnt*-8A lacks the correct expression pattern to be a strong candidate for the primary axis-promoting signal (Bouillet et al. 1996), although mouse *Wnt*-8A, like chicken *Wnt*-8C (Hume and Dodd 1993), is expressed in intriguing sites, including the primitive streak. The generation of null mutations in more mouse *Wnt* genes, in particular *Wnt*-8A, may reveal what role, if any, Wnt genes play in axis formation in vertebrates.

Wnt proteins

Working with Wnt proteins as biological agents has proven to be problematic. There are numerous unpublished tales of failed attempts to produce secreted Wnt proteins in cell culture. In general, overexpression of the genes in cultured cells results in accumulation of misfolded protein in the endoplasmic reticulum (ER; Kitajewski et al. 1992). Secreted forms of Wnt proteins can be found in the extracellular matrix or the cell surface (Bradley and Brown 1990; Papkoff and Schryver 1990; Burrus and McMahon 1995; Schryver et al. 1996), but efforts to solubilize this material have not been successful. Addition of suramin or heparin to cells can lead to a significant increase of Wnt protein in the medium (Bradley and Brown 1990; Papkoff and Schryver 1990), but this protein has not been shown to be biologically active (Papkoff 1989; Burrus and McMahon 1995).

While under any circumstance most Wnt protein is cell bound, several systems have more recently been developed that produce soluble forms. The *Drosophila* Wg (Van Leeuwen et al. 1994) and DWnt-3 (Fradkin et al. 1995) proteins and the mouse Wnt-1 protein (Bradley and Brown 1995) have been recovered from the medium of cultured cells. The amounts secreted are minor, but using in vitro assays for activity, these soluble forms have been shown to be biologically active. Wg protein can be tested for the stabilization of the Arm protein (Van Leeuwen et al. 1994; Fig. 3), and Wnt-1 protein can induce morphological transformation of target cells (Jue et al. 1992; Bradley and Brown 1995). Furthermore, using a hematopoietic stem cell proliferation assay, several Wnt proteins have been shown to be active in solution, and one of these, Wnt-5A, has been partially purified while retaining activity (Austin et al. 1997). These assays for soluble Wnt proteins are critical for defining Wnt protein interactions with other proteins, in particular cell-surface receptors. Moreover, they may lead to the purification to homogeneity of active protein and ultimately to the determination of Wnt protein structure.

Based on interallelic complementation between different *wg* alleles, it has been suggested that the Wg protein consists of different functional domains. These domains apparently have different functions in the patterning of the embryonic cuticle, and they have been suggested to interact with different receptors (Bejsovec and Wieschaus 1995; Hays et al. 1997). Evidence for different domains in Wnt proteins has also emerged from analyzing the phenotype of chimeric Wnt proteins in frog embryos (Du et al. 1995).

The mechanism of Wnt secretion; the role of porc

There are several lines of evidence suggesting that Wnt proteins require specific accessory functions for optimal secretion. The association between overproduced Wnt and Bip proteins (Kitajewski et al. 1992) in the ER indicates that most Wnt protein is misfolded under those conditions. This could be attributable either to a general mishandling of overproduced cysteine-rich proteins or to a limiting concentration of a specific binding partner.

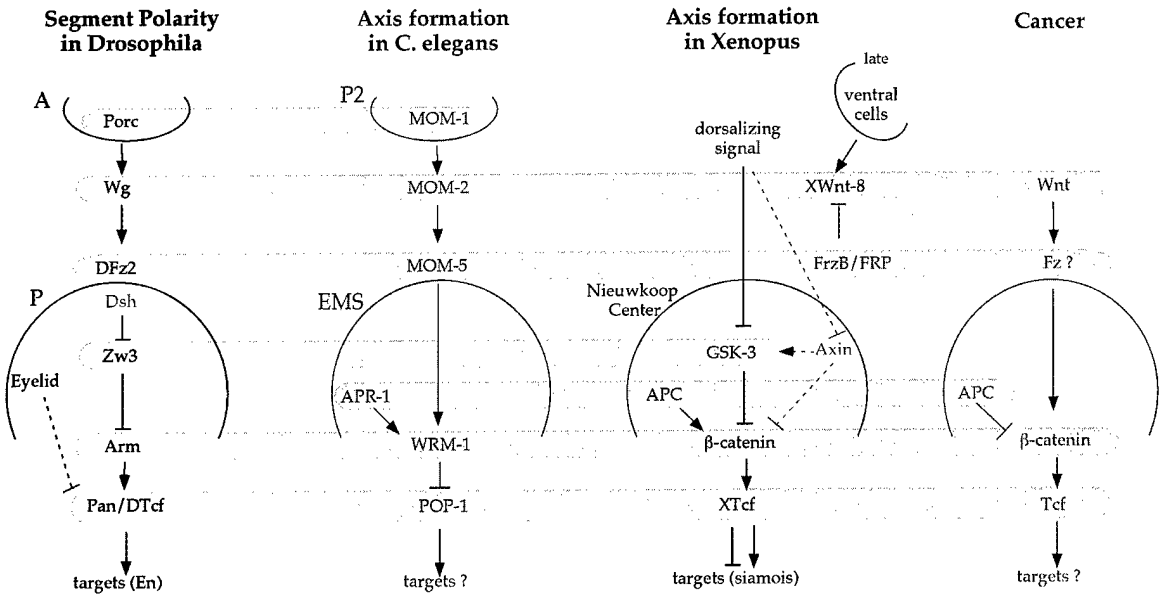

Figure 3. Comparison of Wnt pathways in embryogenesis and carcinogenesis. Related genes are highlighted across the different systems. Potential differences in the pathways are shown in red. Broken lines indicate alternative pathways. During segment polarity in *Drosophila*, anterior (A) cells signal to posterior (P) cells using Wg and the genes shown here and in Fig. 1, resulting in the activation of Arm. There is genetic evidence for an antagonism of Wg signaling by the gene *eyelid*, possibly at the level of DTcf. No role for *Drosophila* adenomatus polyposis coli (APC) in Wg signaling has yet been found. During *C. elegans* embryogenesis, the activity of the Wnt protein MOM-2 in the P2 cell polarizes the E/MS cell and down-regulates nuclear levels of the Tcf-related POP-1 protein. In the target EMS cell, the Fz-related protein MOM-5, the APC homolog APR-1, and the Arm/β-catenin-related WRM-1 protein are required for POP-1 down-regulation. APR-1 is shown acting in parallel to MOM-5 to activate WRM-1, but a direct role in the pathway has not been rule out. Targets of POP-1 have not yet been identified. The *Xenopus* primary axis is specified by a dorsalizing signal that does not appear to be a Wnt or require Dsh, but involves down-regulation of GSK-3, activating β-catenin. Axin could be a direct Wnt signaling component, inhibiting the pathway, possibly by activating GSK-3 or inactivating β-catenin. Axin could be inhibited by the dorsalizing signal or act in parallel. APC can activate the pathway upstream of β-catenin, but its relationship to the other proteins is not clear. XTcf-3 represses expression of the *siamois* gene, but upon binding with β-catenin, activates *siamois*, inducing the formation of the Spemann's organizer. After the onset of zygotic transcription, cells from the Spemann's organizer secrete soluble forms of Fz, called FRP or FrzB, which can counteract the activity of the ventralizing Xwnt-8 signal. In colorectal tumors and some melanomas, mutations in either APC (truncating the protein) or β-catenin (stabilizing it) lead to increased activity of β-catenin/hTcf-4 transcription complexes, which may play a causal role in promoting carcinogenesis. Wnt expression can lead to breast cancer in mice. (See text for more discussion and references.)

The identification of such a putative counterpart may have to await the purification of Wnt in an active form. Initial steps in purifying active Wg protein in our laboratory using sizing chromatography show that the secreted form is considerably larger than monomeric Wg. This may imply that Wg is secreted as a multimer of itself or in a complex with another molecule. Although either explanation is possible, Wg is not linked by disulfide bridges to possible other components, because under nonreducing denaturing gel electrophoresis, Wg runs as a monomer (C. Harryman Samos and R. Nusse, unpubl.).

A genetic clue that Wg secretion requires a specific accessory function is the phenotype of the segment polarity gene *porc*. Embryos mutant for *porc* have the same phenotype as *wg* mutants, and *porc* is required for Wg signaling in larval tissues as well (Cadigan and Nusse 1996; Kadowaki et al. 1996). Like *wg*, *porc* mutant clones behave noncell autonomously, indicating a role in producing the Wg signal (Kadowaki et al. 1996). In contrast to the diffuse staining of Wg protein seen in wild-type embryos, Wg in *porc* mutants is confined to the producing cells (van den Heuvel et al. 1993). The *porc* gene encodes a protein with eight transmembrane domains and is located perinuclearly in transfected cells (Kadowaki et al. 1996). Overexpression of Porc and Wg simultaneously changes the Wg glycosylation pattern but does not lead to increased Wg secretion (Kadowaki et al. 1996). These observations all suggest that Porc has a function within the secretory pathway to facilitate Wg synthesis or processing.

In worm embryos, *mom*-1 encodes a Porc-like protein (Rocheleau et al. 1997). Because it is required in the same cell (the P2 blastomere) as the Wnt gene *mom*-2 (Thorpe et al. 1997), it may have a similar relationship with *mom*-2 as *porc* does with *wg*. In addition, *mom*-3 is also required only in the P2 cell (Thorpe et al. 1997). It has not yet been cloned but may be an additional factor required for Wnt processing or secretion.

Although Porc or MOM-1 is, respectively, required for Wg and MOM-2 secretion, it is not known whether they

are required for other members of the Wnt family. The role of Porc in the secretion or function of other Wnt proteins has not yet been looked at, but the data from *C. elegans* suggest that at least one *Wnt* besides *mom*-2 may require *mom*-1 and *mom*-3 for normal function. These genes have a highly penetrant defect in vulva formation that is not seen in *mom*-2 mutants that appear to be null (Thorpe et al. 1997). This suggests that *mom*-1 and *mom*-3 are required for the production of another worm Wnt protein in the vulva.

Wnt proteins as morphogens

Secreted Wnt proteins can in principle pattern cells over long distances. How far they actually travel from producing cells is difficult to determine because of the poor antigenicity of most Wnt proteins, but for Wg, where good antibodies are available, the protein can be found several cell diameters from the site of synthesis (Van den Heuvel et al. 1989; González et al. 1991; Neumann and Cohen 1997a). Consistent with this, *wg* mutants have patterning defects over a greater area than encompassed by its RNA expression domain. It has been suggested that Wg acts as a morphogen in several tissues (Struhl and Basler 1993; Hoppler and Bienz 1995; Lawrence et al. 1996), that is, it can alter gene expression in a concentration-dependent manner, eliciting different responses at various distances from the Wg-secreting cells. These studies have not adequately ruled out the possibility of a relay mechanism where Wg acts on these cells indirectly, perhaps by activating the expression of another secreted factor, which then patterns cells at a distance.

Two recent papers appear to have settled this debate, at least in the developing wing blade, where Wg has both short- and long-range targets (Zecca et al. 1996; Neumann and Cohen 1997b). A relay mechanism was ruled out by engineering patches of cells to express normal Wg, a membrane tethered form of Wg, or a constitutively activated Arm protein (Zecca et al. 1996; see section on Arm below). Although Wg could activate target genes at a distance from the site of synthesis, the membrane-bound form only works on immediately adjoining cells and the activated Arm could only act cell autonomously, that is, within the cells expressing the construct. The expression pattern of target genes in wings containing Wg-expressing clones and experiments where Wg was partially inactivated were all consistent with the morphogen model, where the shorter-range targets require more Wg activity than the longer-range ones for activation. Wg also activates gene expression noncell autonomously in leg and eye discs (Zecca et al. 1996; Lecuit and Cohen 1997), so Wg may, in general, act as a morphogen.

Whether other Wnt proteins act in vivo as long-range patterning molecules is less clear. One of the best-characterized *Wnt* phenotypes in the mouse is the absence of a large part of the midbrain in *Wnt*-1 mutant animals (McMahon and Bradley 1990; Thomas and Capecchi 1990). Although *Wnt*-1 is initially expressed in the midbrain, expression becomes restricted to a narrow band at the midbrain–hindbrain junction (Wilkinson et al. 1987). Possibly, *Wnt*-1 controls patterning of the CNS beyond its expression domain. The mouse *engrailed-1* (*en-1*) gene is normally expressed in a similar pattern as *Wnt*-1 and its expression decays in a *Wnt*-1 mutant, suggesting that it is a target of *Wnt*-1 signaling (McMahon et al. 1992). When *en*-1 is placed under the control of the *Wnt*-1 promoter, this transgene can significantly rescue the *Wnt*-1 midbrain defect (Danielian and McMahon 1996). This suggests that if there is a nonautonomous action of *Wnt*-1 in the brain, it occurs through a relay mechanism. Likewise, in *Xenopus*, the Wnt signaling pathway appears to induce axis formation in a sequential way, inducing the formation of the Nieuwkoop organizer (Fig. 3), which then secretes factors that induce dorsal mesoderm and notochord (He et al. 1995; Lemaire et al. 1995; Wylie et al. 1996). Clearly, the ability of Wnt proteins to act as morphogens must be examined on a case-by-case basis.

Fz proteins act as receptors for Wnt proteins

For a long time, a significant gap in understanding the mechanism of Wnt signaling was the lack of receptors. The difficulties in generating sufficient quantities of soluble and pure Wnt protein have precluded the identification of specific cell-surface receptors using conventional methods, such as cDNA expression cloning. Recently, however, a series of genetic, cell biological, and biochemical experiments have provided good evidence that members of the Fz family of cell-surface proteins function as receptors for Wnt proteins. *fz* genes encode seven transmembrane receptor-like proteins with an amino-terminal extension rich in cysteine residues that is predicted to be positioned outside of the cell (Figs. 4 and 5; Vinson et al. 1989).

In *Drosophila*, mutations in the first discovered *fz* gene display a tissue or planar polarity defect. In normal wings, the epithelial cells comprising the wing blade are all aligned similarly, so that the wing hairs, one of which is secreted by each cell, all point in a distal direction (Adler 1992). Flies mutant for null alleles of *fz* are viable, but the alignment of epithelial cells is disrupted, resulting in wing hairs pointing in several directions (Vinson and Adler 1987). *fz* mutants also have disruptions in the direction of bristles on the notum and legs (Adler 1992), and in the orientation of the ommatidia comprising the insect compound eye (Zheng et al. 1995). This phenotype is also associated with several other mutations (Wong and Adler 1993; Strutt et al. 1997), including *dsh* (Theisen et al. 1994; Krasnow et al. 1995), which is required for Wg signaling. This raises the possibility that Fz-like molecules might be involved in Wnt reception.

A *fz*-related gene in *Drosophila*, *Dfz2*, is a good candidate for being a specific receptor for Wg. In assays using soluble Wg protein, various cell lines transfected with *Dfz2* bind Wg on their cell surface (Bhanot et al. 1996). Moreover, stable transfection of *Dfz2* into cells that are nonresponsive to Wg (and do not normally express *Dfz2*) confers upon these cells the ability to accumulate Arm protein in a Wg-dependent manner. However, Wg pro-

Figure 4. {*See facing page for legend.*}

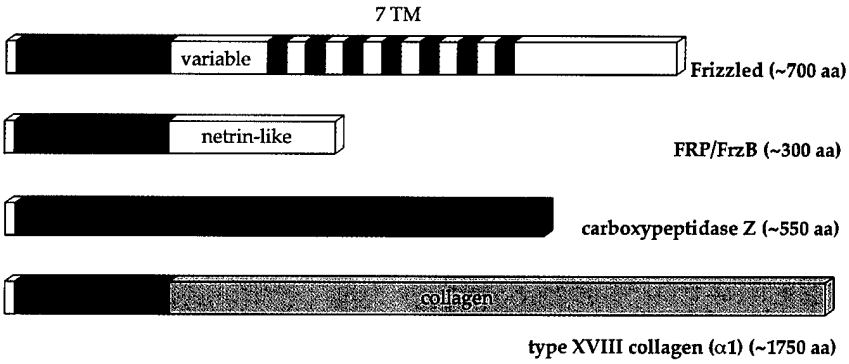

Figure 5. Schematic structures of proteins containing related Fz cysteine-rich domains (CRDs). In addition to the CRDs, the Fz proteins contain seven transmembrane (TM) domains; the FRP/FrzB molecules have some homology to netrins, and the protease carboxypeptidase has an enzymatic domain. A special subtype of collagens also has a CRD domain.

tein does not uniquely bind to cells expressing *Dfz2*; a variety of other Fz family members (Wang et al. 1996; Y.K. Wang et al. 1997), including the original Fz, also enable cells to interact with Wg (Table 3; Bhanot et al. 1996). Because binding affinity cannot be measured in these assays, there is no information on the relative strengths of these interactions. There is at present no mutant in the *Dfz2* gene, so it is possible that the gene is not required for Wg signaling in vivo and that Wg uses another receptor. Still, the demonstration that Dfz2 can bind and transduce the Wg signal in cell culture makes it an attractive candidate for a Wg receptor.

Genetic evidence for fz–Wnt *interactions in the worm*

The genetic evidence that Wnt proteins require Fz proteins for signaling, although lacking in flies, is accumulating in *C. elegans* (Table 3), though the story is a little complicated. Mutations in the *lin*-17 gene, which encodes a Fz protein (Sawa et al. 1996), affect the same cells that are influenced by the *Wnt* gene *lin*-44 (Herman et al. 1995), but with significant differences. Although *lin*-44 mutants have reversals of polarity in certain cells undergoing asymmetric divisions (Herman et al. 1995), these same cells in *lin*-17 mutants undergo symmetrical divisions; that is, polarity is completely lost (Sawa et al. 1996). One model to explain this is that there is a second signal, possibly another *Wnt*, which also works through the *lin*-17 receptor. Thus, the *lin*-17 phenotype is predicted to be the sum of defects in two signals.

In the EMS cell fate decision controlled by the *mom* genes, the *mom*-5 gene encodes a Fz member (Rocheleau et al. 1997), genetically interacting with the Wnt protein MOM-2. However, the penetrance of a null allele of *mom*-5 is very low (<10%), whereas the penetrance of the strong *mom*-2 *Wnt* mutations is >70%. This discrepancy could be explained by the presence of another *fz* gene. However, *mom*-2;*mom*-5 double mutants have a penetrance of only 8%, suggesting that *mom*-5 may in part play a negative role in *mom*-2 signaling. On the other hand, both *mom*-2 and *mom*-5 have a second defect (in the orientation of the mitotic spindle of the ABar blastomere at the eight-cell stage; Fig. 2) in which both genes have identical phenotypes with 100% penetrance (Rocheleau et al. 1997; Thorpe et al. 1997). Despite the complications, the story from the worm so far suggests a close relationship between Wnt and Fz proteins.

A third example of Wnt–Fz interactions in *C. elegans* is between *egl*-20 (a *Wnt* gene; C. Kenyon, pers. comm.) and *lin*-17; both genes are required in the migration of the neuronal Q cell (Harris et al. 1996).

Other Fz–Wnt interactions

Experiments in *Xenopus* also demonstrate that Fz molecules can transduce Wnt signals. Coinjection of a rat Fz with XWnt-8 leads to relocation of the Wnt protein from the ER to the cell surface, presumably because of binding to the receptor (Yang-Snyder et al. 1996). Fz-injected embryos also become more sensitive to the axis-duplicating activity of injected Wnt proteins. One Wnt member, XWnt-5A is normally unable to produce a secondary axis, but when coinjected with human fz5, this response is elicited (He et al. 1997). This result indicates that *Xenopus* normally does not express the cognate Fz receptor for XWnt-5A. However, in the absence of exogenous Fz, XWnt-5A has an effect: it can block the axis-inducing activity of XWnt-8 (Torres et al. 1996). It is not clear how XWnt-5A mediates this inhibitory effect, though a decrease in cell adhesion was implicated, nor is it known whether a Fz member is involved in this activity.

Somewhat ironically, a ligand for the original Fz protein in *Drosophila* is not known. In vitro, the Wg protein can bind to Fz (Bhanot et al. 1996), but *wg* does not appear to have a tissue polarity phenotype (though the pleiotropy of *wg* mutations makes this difficult to test rigorously). Clonal analysis of *fz* has revealed a puzzling

Figure 4. Sequence comparison between various members of the Fz protein family: *Drosophila* Fz; *Drosophila* Fz2 (DFz2); two mouse proteins (mfz8 and mfz3); two human proteins (hfz5 and FZD3); the *C. elegans* LIN-17 protein; and the *Drosophila* Smoothened (Smo) protein. Cysteine residues are in cyan throughout. Absolutely conserved residues are in magenta, and residues conserved in at least 6/8 protein are in green. The positions of the cysteine-rich domain, the nonconserved linker domain, and the seven transmembrane domains (TM) are indicated above the alignment. The type 2 angiotensin II receptor (rAT2R) has a short stretch of weak homology with the Fz proteins (Mukoyama et al. 1993), which is indicated.

Table 3. *Interactions between Fz and Wnt proteins*

fz	Species	Wnt interaction	Type of interaction
fz	*Drosophila*	Wg	binding
fz2	*Drosophila*	Wg	binding
lin-17	*C. elegans*	*lin*-44	genetic
lin-17	*C. elegans*	*egl*-20	genetic
mom-5	*C. elegans*	*mom-2*	genetic
fz1	rat/*Xenopus*	XWnt-8	binding
fz4	mouse	Wg	binding
fz7	mouse	Wg	binding
fz8	mouse	Wg	binding
fz5	human	Wg	binding
		XWnt-5A	axis induction in *Xenopus*
FZD3	human	Wg	binding

and interesting phenomenon termed directional nonautonomy, where cells outside the clone also display the mutant phenotype (Vinson and Adler 1987). These phenotypically mutant cells outside the clone are usually distal to the fz^- cells. One explanation for this effect is that the Fz ligand, possibly a Wnt, moves over the field of cells in a proximal to distal direction, but cannot traverse the *fz* clone. In the eye, the data are consistent with the polarity signal emanating from the equator (Zheng et al. 1995). Identification of the tissue polarity ligand(s) may shed light on this intriguing problem.

Are Fz proteins the only Wnt receptors?

The above studies make a compelling case that Fz proteins are required for Wnt reception, but are they sufficient? There is a requirement for sulfated proteoglycans for Wnt signal transduction (see below), perhaps acting as a coreceptor, analogous to the relationship between proteoglycans and fibroblast growth factor (FGF) receptors (Schlessinger et al. 1995). The cell-surface receptor Notch has been proposed to play a role in Wg signaling based on somewhat complicated genetic interactions (Couso and Martinez Arias 1994), but complete removal of Notch activity in the wing and embryo does not reveal a defect in Wg signaling (Rulifson and Blair 1995; Cadigan and Nusse 1996). Finally, the Smoothened (Smo) protein, a distantly related member of the *fz* gene family (Alcedo et al. 1996; Van Den Heuvel and Ingham 1996) can associate with the multiple transmembrane protein Patched (Ptc), to constitute a functional Hedgehog (Hh) receptor (Fig. 1; Stone et al. 1996). In this complex, Hh binds to Ptc (Marigo et al. 1996; Stone et al. 1996), but the Smo protein is thought to transduce the signal. Whether Smo has a separate ligand is not known [Wg protein does not bind to Smo-transfected cells (Nusse et al. 1997)], nor is it clear whether Ptc-related molecules interact with other Fz proteins. Further biochemical characterization of the Fz proteins is needed to clarify this issue.

FRP, FrzB, and other secreted forms of Fz proteins

In addition to the integral membrane Fz proteins described above, *Xenopus* and other vertebrates produce several secreted proteins (called FrzB or FRP), which consist of a cysteine-rich domain (CRD) very similar to those in Fz molecules, followed by a stretch of charged residues containing a short stretch of homology to the netrins (Fig. 5; Shirozu et al. 1996; Finch et al. 1997; Leyns et al. 1997; Rattner et al. 1997; S.W. Wang et al. 1997). At least one of these molecules, FrzB or FRP, is specifically expressed in the Spemann's organizer in *Xenopus* embryos, where it can function as an antagonist of XWnt-8, a ventralizing factor (Leyns et al. 1997; S.W. Wang et al. 1997). Antagonism is probably mediated by direct binding of XWnt-8 to the CRD of the FRP proteins. Whether all FRPs function to down-regulate Wnt protein function is not clear; one can also imagine that they promote Wnt secretion or otherwise function in the distribution of these ligands.

In addition to the FRP and Fz proteins, the CRD motif is found in two other proteins (Fig. 5): carboxypeptidase Z (Song and Fricker 1997); and several isoforms of type XVIII collagen (Rehn and Pihlajaniemi 1995). The function of these domains is not clear, nor is it known whether these molecules can bind to Wnt proteins.

The requirement for proteoglycans in Wnt signaling

The binding of Wnt proteins to proteoglycans such as heparin has long been noted, and more recently, several lines of evidence suggest that this interaction has physiological relevance. Two *Drosophila* genes with embryonic mutant phenotypes very similar to *wg* have been shown recently to encode, respectively, homologs of UDP–glucose dehydrogenase (*sugarless;* Binari et al. 1997; Häcker et al. 1997; Haerry et al. 1997) and *N*-deacetylase/*N*-sulfotransferase (X. Lin and N. Perrimon, in prep.), which are required for heparin sulfate biosynthesis. The *sugarless* mutant phenotype was partially rescued by injection of embryos with heparin sulfate, and injection of heparinase into wild-type embryos created *wg*-like phenotypes. Null alleles of *sugarless* strongly reduced but did not completely block Wg signaling (Häcker et al. 1997; Binari et al. 1997). This is in contrast to genes such as *dsh* and *arm*, which are absolutely required for Wg signaling even when Wg is grossly overexpressed (Noordermeer et al. 1994; Manoukian et al. 1995). These mutants provide in vivo evidence for the importance of sulfated proteoglycans in Wg function, although their exact role is unclear.

Sulfated glycosaminoglycans are required for soluble Wg to optimally stabilize Arm in a *Drosophila* cell line, and the addition of exogenous heparin can enhance Wg signaling in this assay (Reichsman et al. 1996). These results suggest a role for these proteoglycans in either binding of Wg to cells or the transduction of the signal.

Interestingly, heparin sulfates are not required for Wg binding to DFz2 (Bhanot et al. 1996), although a decrease in affinity cannot be ruled out. Removal of heparin sulfate (via heparinase) has been shown to block XWnt-8 activity in animal cap assays in *Xenopus* (Itoh and Sokol 1994), suggesting that proteoglycans are a general requirement for Wnt signaling.

The mechanism of signal transduction by Fz proteins

The Fz receptors include seven transmembrane domains, an amino-terminal extension acting as a ligand binding domain in DFz2 (Bhanot et al. 1996), and a cytoplasmic tail (Figs. 4 and 5). The fact that almost all previously identified seven transmembrane receptors utilize G proteins for signaling suggests that Fz molecules may as well. At present, however, there is only circumstantial evidence for a G protein in Wnt or Fz signaling. Dsh and another potential component of Wnt signaling, Axin, both contain domains that are found in G-protein regulators (see below), but thus far there is no genetic or biochemical evidence for a G protein in any Wnt pathway. In addition, there is no sequence homology between Fz proteins and the known G protein-coupled receptors, save for a short stretch of similarity with the type II angiotensin 2 receptor in the third cytoplasmic loop (Fig. 4; Mukoyama et al. 1993). The importance of this homology is not clear, although it may be significant to note that G proteins in general are known to interact with the third cytoplasmic loop of their cognate receptors (Bourne 1997). These issues can now be addressed through site-directed mutagenesis of Fz proteins, followed by in vitro or in vivo characterization.

It has also been recognized that the carboxyl terminus of many Fz proteins contains a motif (SXV) that can interact with PDZ domains (Fig. 4). Dsh has a PDZ domain and is the first known component of Wg signaling downstream of the receptor, but experiments in our laboratory have not revealed a direct interaction with any Fz proteins (Nusse et al. 1997). This may not be surprising in light of the report demonstrating that only some PDZ domains bind to the SXV motif (Songyang et al. 1997), and the Dsh PDZ falls into the nonbinding class (Doyle et al. 1996; Morais Cabral et al. 1996). The importance of the SXV tail for Fz function is also put into question by the result that replacing it with a GFP moiety does not affect signaling in the case of *lin*-17 in the worm (Sawa et al. 1996). In summary, how Fz proteins transduce Wnt signals to the inside of the cell remains an open question.

Signaling downstream of the receptor

dsh

In the genetic Wg signal transduction pathway, *wg* activates *dsh* (presumably through a Fz) which in turn inhibits *zw3*. *dsh* encodes a cytoplasmic protein (Klingensmith et al. 1994; Theisen et al. 1994) that has highly conserved counterparts in *Xenopus* and mouse, in particular in the amino terminus and in the central PDZ-containing domain (Fig. 6). In flies, *dsh* is required for Wg signaling in many tissues (Couso et al. 1994; Klingensmith et al. 1994; Theisen et al. 1994; Park et al. 1996a; Lecuit and Cohen 1997; Neumann and Cohen 1997b). Overexpression of Dsh can mimic Wnt signaling in *Drosophila* and *Xenopus* (Rothbacher et al. 1995; Sokol et al. 1995; Yanagawa et al. 1995; Axelrod et al. 1996; Cadigan and Nusse 1996; Park et al. 1996a). In mice, however, a knockout of a *dsh* gene did not display any of the dramatic developmental defects associated with Wnt proteins, though behavioral and neurological abnormalities were observed (Lijam et al. 1997). There are several other mouse *dsh* genes, all widely expressed (Klingensmith et al. 1996) and it seems likely that they act in a redundant manner. Thus far, no mutations in worm *dsh* genes have been reported.

It is not known how Dsh proteins work as Wnt signal-transducing components, but over the past few years, several motifs have been identified in these proteins. A picture is emerging of Dsh proteins being modular proteins that can interact with various other signaling components. Besides the PDZ domain discussed above, Dsh proteins contain two other domains found in proteins participating in G-protein-mediated signaling. The Axin protein, a negative regulator of Wnt signaling (Zeng et al. 1997) that contains a RGS (regulators of G-protein signaling) motif (Koelle 1997), shares a region of homology with Dsh proteins that we refer to as DIX (Fig. 6). Dsh proteins lack a RGS, but their carboxyl ends contain a so-called DEP domain (Ponting and Bork 1996), found in a variety of proteins, many of which participate in G-protein signaling.

The *Drosophila* Dsh is a phosphoprotein localized predominantly in the cytoplasm of the cell and not in the nucleus (Yanagawa et al. 1995). Wg stimulation in cells or embryos leads to hyperphosphorylation of Dsh (Yanagawa et al. 1995). It is not clear which protein kinase catalyzes this phosphorylation, although the Dsh protein can be found in a complex with casein kinase II (CKII, Willert et al. 1997) and is phosphorylated by CKII in vitro. Possibly, the hyperphosphorylated form of Dsh is the active form, and phosphorylated Dsh transduces the signal onto the next signaling component, directly or indirectly leading to the inhibition of Zw3. This view is somewhat oversimplified in light of the finding that under certain conditions, that is, overexpression of DFz2 in the absence of Wg, Dsh becomes hyperphosphorylated but does not activate the pathway (Willert et al. 1997). It may be that the phosphorylation pattern is not identical to that in Wg-stimulated cells, or it could be that hyperphosphorylation of Dsh is necessary but not sufficient for the signal to be transduced. Identification of more binding partners of Dsh will hopefully shed more light on its mechanism of action.

zw3/GSK-3

Flies mutant for *zw3* (Peifer et al. 1994b; Siegfried et al. 1994) and frog embryos expressing dominant-negative

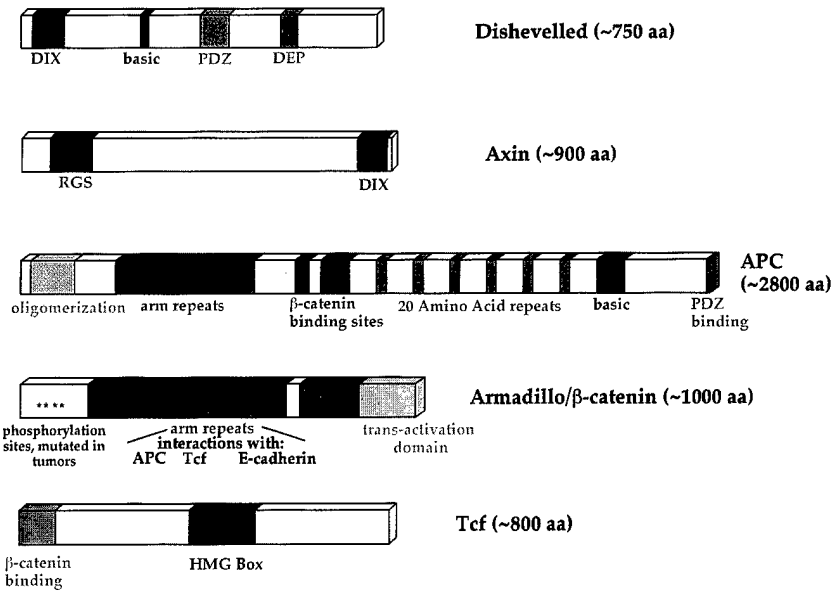

Figure 6. Schematic structure of intracellular Wnt signaling components. Dsh contains a domain also found in Axin (which we call the DIX domain), a conserved basic stretch, a PDZ (previously called GLGF or DHR) domain (Ponting et al. 1997), and a DEP (Dsh/egl-10/pleckstrin domain; Ponting and Bork 1996), the latter found in various proteins that interact with G proteins. Axin has an RGS motif (Koelle 1997) and a DIX domain. APC has seven Arm repeats, three β-catenin-binding sites, a set of internal repeats, and a basic domain. APC has a motif at the carboxyl terminus that can interact with PDZ domains (Matsumine et al. 1996). The Arm/β-catenin molecule has an amino terminus that regulates stability through several serine residues (asterisks). In addition to the internal Arm repeats, the protein has a transcriptional activator domain at the carboxyl terminus. The Tcf has a domain interacting with β-catenin and a HMG box-like DNA-binding domain.

versions of its vertebrate counterpart GSK-3 (Dominguez et al. 1995; He et al. 1995; Pierce and Kimelman 1996) both have phenotypes consistent with the constitutive activation of Wnt pathways through Arm/β-catenin. This has led to a model where Wnt acts to negatively regulate the Zw3/GSK-3 kinase, though the data equally support Zw3/GSK-3 acting in parallel as a repressor of Arm/β-catenin. The GSK-3 enzyme has been characterized extensively in mammalian cells and is unusual in that it is constitutively active in nonstimulated cells (Woodgett 1991). The enzyme activity can be down-regulated by the addition of insulin or epidermal growth factor (EGF) to serum-starved cells and is correlated with phosphorylation on residue Ser-9, probably via protein kinase rsk-90 or protein kinase B (Stambolic and Woodgett 1994; Cross et al. 1995). Thus, there is precedence for the idea that Wnt proteins inhibit Zw3/GSK-3 through covalent modification.

Cook et al. (1996) found that the addition of soluble Wg protein to the mammalian cell line C3H10T1/2 results in an approximate twofold down-regulation of GSK-3 activity in cell extracts. They showed that this effect was pharmacologically distinct from insulin and EGF-mediated inhibition of the kinase. A phorbol ester-sensitive protein kinase C (PKC) was shown to be required for the Wg effect. PKC is known to phosphorylate GSK-3 in vitro, lowering its activity (Goode et al. 1992). Identification of the in vitro phosphorylation sites of PKC on GSK-3 should allow the testing of the importance of these sites for in vivo regulation.

The fact that the reduction of GSK-3 activity upon stimulation with Wg is only 50% is cause for some concern (Cook et al. 1996). However, other inhibitory signals, such as insulin, inhibit to roughly the same degree. Is a twofold reduction of GSK-3 sufficient to transduce the Wnt signal, stabilizing β-catenin? This was not examined, but if Wg does cause the accumulation of β-catenin in these cells, would insulin do so as well? If the answer is no, this raises several interesting possibilities, such as different intracellular pools of enzyme, with only the Wnt-sensitive pool able to regulate β-catenin.

A complex among Zw3/GSK-3, Arm/β-catenin, and adenomatous polyposis coli

The Arm protein is similar to vertebrate β-catenin and plakoglobin, proteins binding to E-cadherin (McCrea et al. 1991) and linking adhesion complexes to the cytoskeleton. Arm/β-catenin proteins contain a set of 12 internal repeats, the structure of which was recently solved (Huber et al. 1997). Each repeat consists of 3 helices and the 12 repeats together form a superhelical, protease-resistant rod that contains a long, positively charged groove. This groove is suggested to be important in the binding of Arm/β-catenin to its various partners: cadherin, adenomatous polyposis coli (APC), and Tcf (see below; Fig. 6).

Although essential for cellular adhesion, *arm* mutants were first identified because of their *wg*-like phenotype in embryos (Wieschaus and Riggleman 1987). These mutations are carboxy-terminal truncations, leaving the internal repeats and cadherin-binding domains intact (Fig. 6). Alleles of *arm* disrupting the cadherin-binding domains show the expected cell adhesion defect (Orsulic and Peifer 1996). Antisense and overexpression experiments in *Xenopus* are also consistent with β-catenin playing a role in Wnt signaling in addition to its role in cell adhesion.

Wnt signaling regulates Arm/β-catenin levels post-transcriptionally in flies (Riggleman et al. 1990; Orsulic and Peifer 1996) and frogs (Larabell et al. 1997), leading to

cytoplasmic and nuclear accumulation. This increase in Arm/β-catenin is attributable to increased stability in the presence of Wnt signaling (Hinck et al. 1994; Van Leeuwen et al. 1994). Consistent with its proposed place in the pathway, inactivation of zw3/GSK-3 also causes this accumulation (Peifer et al. 1994b; Stambolic et al. 1996; Yost et al. 1996; Larabell et al. 1997). The stabilized Arm protein is underphosphorylated compared to membrane-bound, cadherin-associated protein (Peifer et al. 1994a; Van Leeuwen et al. 1994). Thus, the simplest model would suggest that Zw3/GSK-3 directly phosphorylates Arm/β-catenin, destabilizing it, probably by promoting its entry into the ubiquitin–proteasome degradation pathway (Aberle et al. 1997). There are four potential GSK-3 phosphorylation sites in the amino-terminal portion of β-catenin that are conserved in Arm. Mutation of these sites led to a β-catenin that is more stable than wild-type β-catenin and considerably more potent in secondary axis formation in *Xenopus* (Yost et al. 1996). Deletions at the amino terminus of Arm (removing the four serine/threonine residues) result in a constitutively active Arm protein (Zecca et al. 1996; Pai et al. 1997). Both in flies and *Xenopus*, these mutant proteins are no longer sensitive to Zw3/GSK-3 regulation (Yost et al. 1996; Pai et al. 1997).

Although the above results make a compelling case for the importance of the amino-terminal phosphorylation sites in regulating Arm/β-catenin stability and signaling activity, the data that Zw3/GSK-3 is the direct kinase are less convincing. GSK-3 can phosphorylate β-catenin in vitro, and the activated form of β-catenin lacking the putative GSK-3 sites is phosphorylated less efficiently in vitro and in vivo (Yost et al. 1996). However, these assays were not quantitative, and the in vitro phosphorylation did not appear to be stoichiometric. In addition, other groups have not found Arm/β-catenin to be phosphorylated by Zw3/GSK-3 (Rubinfeld et al. 1996; Stambolic et al. 1996; Pai et al. 1997). Although there are many technical explanations for these discrepancies, it is also possible that GSK-3 is not the kinase that phosphorylates β-catenin in vivo.

If Zw3/GSK-3 does not directly interact with Arm/β-catenin, are there any known proteins that could form the bridge? An interesting candidate is the product of the adenomatous polyposis coli (*APC*) gene (for review, see Polakis 1997), mutations in which are correlated with colorectal cancer (see below). Tumor cell lines producing truncated forms of APC protein have high levels of cytosolic β-catenin because of increased stability (Rubinfeld et al. 1996).

Transfection of these cells with full-length APC or with fragments of the protein that are missing in the truncated forms reduces the β-catenin levels dramatically (Munemitsu et al. 1995). These tumor cell lines have been found to have complexes of GSK-3, β-catenin, and APC (Rubinfeld et al. 1996). The percentage of each protein in this complex is not clear, but GSK-3 is enriched in the β-catenin pool that also bound APC. GSK-3 was found to stoichiometrically phosphorylate an APC fragment, which stimulated its binding to β-catenin (Rubinfeld et al. 1996). These data suggest the possibility that GSK-3 destabilizes β-catenin through phosphorylation of APC, promoting APC binding to β-catenin and precipitating β-catenin degradation.

A positive role for APC in Wnt signaling

The data summarized above suggest that if APC plays a role in Wnt signaling, it would be a negative regulator of the pathway. However, some recent experiments are inconsistent with this view. *Xenopus* has an APC homolog (XAPC) that is found primarily in a complex with β-catenin (Vleminckx et al. 1997). Surprisingly, overexpression of XAPC in ventral blastomeres results in the induction of dorsal markers and a second notochord on the ventral side of embryos. This overexpression does not affect endogenous β-catenin levels, but β-catenin is necessary for the axis-inducing activity of XAPC. Fragments of human APC that have been shown to destabilize β-catenin in colon cancer cell lines also efficiently induced secondary axes (Vleminckx et al. 1997). These results suggest that APC has a positive signaling role in the Wnt pathway.

Results from *C. elegans* support the *Xenopus* findings. RNA interference studies, which are thought to specifically inhibit translation of the targeted message, with worm APC (*apr*-1)- and β-catenin (*wrm*-1)-related genes both produced embryos with *mom* phenotypes. The penetrance of the *wrm*-1 phenotype was 100%, but the *apr*-1 *mom* phenotype occurred only 26% of the time. When APR-1 interference was performed in a *mom*-2 (*Wnt* gene) or *mom*-5 (*fz*-related gene) background (which had 39% and 8% penetrance, respectively), 100% of the embryos lacked E cells (Rocheleau et al. 1997). This was taken as evidence that *mom*-2 and *apr*-1 act in parallel, converging at *wrm*-1, but these results can also be explained by another unidentified *Wnt* and *APC*-like gene acting redundantly with *mom*-2 and *apr*-1. In any case, once again APC is implicated positively in Wnt signaling.

Can the tumor cell culture results—where APC's primary role appears to be to stimulate β-catenin degradation—be reconciled with the frog and worm data? Perhaps APC, phosphorylated by Zw3/GSK3, binds to β-catenin and promotes its degradation. Upon Wnt stimulation, the nonphosphorylated form of APC still binds to β-catenin, promoting β-catenin signaling. In mammalian cells constitutively expressing Wnt-1, there is an increase in APC levels and in the stability of APC/β-catenin complexes, compared to untransfected cells (Papkoff et al. 1996). Further analysis of this effect in cell lines with inducible Wnt expression or after addition of soluble Wnt should help clarify the relationship between Wnt and APC.

An APC homolog in flies has also been identified (Hayashi et al. 1997). This protein can stimulate β-catenin turnover in colorectal tumor cell lines. However, embryos homozygous for a large deficiency removing the gene show no defect in Arm distribution (Hayashi et al. 1997). The analysis of additional mutations within this

gene should be informative, in addition to testing for its phenotype in the absence of maternal contributions.

Binding of Arm/β-catenin to Tcf–LEF-1

At the same time that it was being appreciated that Arm and β-catenin accumulate in the nucleus after Wnt stimulation, several groups reported that β-catenin could bind to HMG box transcription factors of the Tcf–LEF-1 family. Coexpression of β-catenin and these proteins resulted in accumulation of β-catenin in the nucleus. Tcf and LEF-1 proteins were found originally as enhancer binding factors for T cell-specific genes (Clevers and Grosschedl 1996). Binding of Tcf proteins to DNA results in bending of the helix (Giese et al. 1992), but by themselves, these proteins are poor transcriptional activators. However, complexes between Tcf proteins and β-catenin act as potent transcriptional activators of reporter gene constructs containing the DNA element recognized by Tcf (Molenaar et al. 1996; Korinek et al. 1997; Morin et al. 1997). Overexpression of Lef-1 in *Xenopus* causes an axis duplication that is greatly enhanced by coinjection of β-catenin, whereas dominant-negative forms (that can bind DNA but not β-catenin) are able to block the formation of the primary and the Wnt-induced secondary axes (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996). The Tcf proteins have finally provided the link between Wnt signaling and transcriptional regulation.

Similar results—but underpinned by loss-of-function genetics—were obtained with a *Drosophila* homolog of Tcf, which is also named *pan* (Brunner et al. 1997; Van de Wetering et al. 1997). The DTcf protein binds to Arm (Brunner et al. 1997; Van de Wetering et al. 1997). Null mutations in DTcf cause a *wg*-like segment polarity phenotype, and a conditional allele can give defects in adults similar to *wg*. Genetically, DTcf mutations are downstream of Arm, consistent with the vertebrate results (Brunner et al. 1997; Van de Wetering et al. 1997).

In *C. elegans*, *pop*-1 encodes a protein with a HMG box, suggesting that it has a function similar to Tcf proteins (Lin et al. 1995). *pop*-1 is involved in the *Wnt*-dependent asymmetric cell division of the EMS blastomere referred to earlier. However, unlike *Drosophila*, where *wg* and *Dtcf/pan* have similar phenotypes, *pop*-1 has the opposite phenotype as the Wnt components of the *mom* class described above. Genetically, *pop*-1 is downstream of all the *mom* genes and the β-catenin-like gene *wrm*-1 (Rocheleau et al. 1997). The POP-1 protein is post-transcriptionally down-regulated by the Wnt pathway in the nuclei of the EMS daughter closest to the Wnt-producing P2 cell (Fig. 2; Rocheleau et al. 1997). The mechanism of this repression in not understood.

Is the *pop*-1 mutant phenotype evidence that Wnt signaling in worms is fundamentally different from flies and frogs? A few points in this regard need to be emphasized before reaching this conclusion. First, the conserved amino-terminal domain of the fly and vertebrate Tcf proteins (which binds Arm/β-catenin) is not found in POP-1 (Lin et al. 1995; Van de Wetering et al. 1997) so it may not even be the true Tcf worm counterpart. On the other hand, WRM-1 is distantly related to Arm and β-catenin, so perhaps WRM-1 and POP-1 can bind each other. This obviously needs to be tested directly. Second, the possible regulation of Tcf protein distribution by Wnt proteins has not yet been examined in flies or frogs, so it is not clear that Wnt-dependent down-regulation of POP-1 protein seen in the worm is unique. Finally, there are recent data on a target gene of the Wnt pathway in *Xenopus*, *siamois*, suggesting that the function of *pop*-1 in worm Wnt signaling may not be that different from the situation in frogs.

Several lines of evidence suggest that the homeobox gene *siamois* is a major target of β-catenin/XTcf-3 action in axis formation in frog embryos (Brannon and Kimelman 1996; Carnac et al. 1996; Fan and Sokol 1997), where it is expressed only on the dorsal side of gastrulating embryos. The siamois promoter contains several XTcf-3 binding sites, which are needed for β-catenin-mediated activation of *siamois* promoter reporter constructs (Brannon et al. 1997). Consistent with endogenous *siamois* expression, the wild-type reporter construct was expressed at low levels when injected ventrally. However, when the XTcf-3 binding sites were mutated, expression on the ventral side was almost as high as the parental construct's expression dorsally. This indicates that in addition to its activating role in conjunction with β-catenin, XTcf represses *siamois* expression in the absence of high levels of nuclear β-catenin (Brannon et al. 1997). Therefore, if the endogenous XTcf were mutated, a dorsalized embryo would be predicted—the opposite of the ventralized phenotype seen in β-catenin-depleted embryos. Thus, if *pop*-1 is a functional Tcf homolog in *C. elegans*, its phenotype may not be that unusual, depending on how important its repressing activity in the absence of Wnt signaling is.

In *Drosophila*, there is also evidence for derepression when the DTcf– binding site is mutated in the Wg response element of the Ultrabithorax (Ubx) promoter, but the effect is minor compared to that seen with *siamois* (Riese et al. 1997). Thus, at least for the few Wg targets so far examined, the activation activity of DTcf outweighs any derepression of target genes in DTcf mutants. More work is needed in all three model systems to determine the commonalities and differences in their Wnt pathways.

Wnt signaling components in cancer

The first *Wnt* gene discovered, mouse *Wnt*-1, was identified by virtue of its ability to form mouse mammary tumors when ectopically expressed due to proviral insertion (Nusse and Varmus 1982). Although the relation between Wnt proteins and mouse breast tumorigenesis has been extended, there is still no direct link between Wnt signaling and human breast cancer. However, APC and β-catenin implicate Wnt signaling in other forms of human cancer.

Mutations in the *APC* gene are found in familial and spontaneous colon carcinomas. As described above, tu-

mor cell lines homozygous for *APC* mutations have abnormally high levels of β-catenin (Munemitsu et al. 1995). β-Catenin forms a complex with one of the human Tcf homologs (hTcf-4), which activates expression of reporter constructs containing hTcf-4-binding sites. Transfection of full-length *APC* into those cells inhibits expression of the reporter gene constructs (Korinek et al. 1997; Morin et al. 1997). Mutant forms of *APC*, which are unable to stimulate degradation of β-catenin, are incapable of blocking target gene expression. Thus, at least one effect of *APC* mutations is to activate a transcriptional complex that may contribute to the cell's oncogenic potential.

This theory is strengthened by the existence of several colon carcinoma cell lines with wild-type *APC* that nevertheless display strong expression of hTcf-4 reporter constructs (Korinek et al. 1997; Morin et al. 1997). These cell lines have mutations in the β-catenin gene similar to the activating mutations created in *Xenopus* and flies. Similar mutations are also present in several melanoma cell lines (Rubinfeld et al. 1997). These findings implicate stable β-catenin as the common feature of most colon carcinomas and many melanomas.

Does this mean that mutations in *APC* only contribute to tumorigenesis through stabilization of β-catenin? Results in cultured cells with expression of amino-terminal deleted versions of β-catenin (which constitutively activate Wnt signaling) show the formation of stable complexes of the mutant β-catenin and APC (Munemitsu et al. 1996; Barth et al. 1997). This raises the possibility that mutations in β-catenin may affect APC functions such as cell migration (Näthke et al. 1996) in addition to promoting Tcf-mediated transcriptional changes. It may be informative to stably transfect colon tumor cell lines with dominant-negative forms of hTcf-4 that cannot bind β-catenin (and presumably do not affect the APC protein) to see if their oncogenic characteristics can be reverted. When this is done with wild-type APC, the tumor cell growth rate is reduced sharply, because of increased apoptosis (Morin et al. 1997). If similar results are obtained with the mutated hTcf-4, this would strongly support hTcf-4 playing a causal role in colon cancer.

Do all Wnt functions work through Arm/β-catenin and Tcf proteins?

The *fz* and *dsh* genes function in the tissue polarity pathway in *Drosophila*, which regulates cell orientation in wings, legs, and eyes (Adler 1992; Theisen et al. 1994; Zheng et al. 1995). Because Fz can bind Wg (Bhanot et al. 1996), it is likely that a Wnt is the physiological polarity signal. However, this signaling pathway does not appear to be a standard Wnt pathway. Several other genes, *fuzzy*, *inturned*, and *rhoA* (Wong and Adler 1993; Park et al. 1996b; Strutt et al. 1997), have tissue polarity phenotypes and appear to act downstream of *fz* and *dsh*, but these genes have no apparent defects in Wg signaling. In addition, there is a *dsh* allele with a strong tissue polarity phenotype but no *wg*-like phenotypes (Theisen et al. 1994). The data suggest that the Wnt and planar polarity pathways branch at *dsh*, though it remains to be demonstrated that *zw3*, *arm*, and *DTcf* play no role in tissue polarity.

In *C. elegans*, it also appears that a branch occurs in a Wnt pathway. As described earlier, there is a signal from the P2 blastomere that polarizes the EMS cell. A *Wnt* gene (*mom*-2) and genes related to *porc* (*mom*-1), *fz* (*mom*-5), *APC* (APR-1), *arm* (WRM-1), and *Tcf* (*pop*-1) are required for this polarization (Rocheleau et al. 1997; Thorpe et al. 1997). A subset of these genes are also needed for the proper orientation of the mitotic spindle of the ABar cell (Fig. 2); *mom*-1, *mom*-2, and *mom*-5 are needed, but no requirement was seen for the others (Rocheleau et al. 1997), suggesting a branch in the pathway downstream of the MOM-5 receptor, perhaps at the as yet unidentified worm *dsh*. It will be interesting to see whether the ABar cell is a target for a signaling cascade similar to the fly planar polarity pathway.

wg *autoregulation*

In the embryonic epidermis, *wg* is required for the maintenance of its own transcription (Hooper 1994; Manoukian et al. 1995; Yoffe et al. 1995). This maintenance requires *porc*, but not *dsh* or *arm* (Manoukian et al. 1995). Unless one argues that *porc* mutants contain less stable Wg transcripts compared to *dsh* and *arm* mutants, embryonic *wg* autoregulation involves a different mechanism than most *wg* functions. In another study, using different double mutant combinations, it was found that *porc* and *dsh* were required for *wg* autoregulation but not *arm* (Hooper 1994). These studies clearly must be extended, hopefully by the identification of the Wg response elements in the Wg promoter.

In contrast to the embryo, *wg* negatively autoregulates its own expression at the dorsal/ventral boundary of the wing imaginal discs (Rulifson et al. 1996). This effect requires *dsh* but not *arm*. The Notch protein, which is the receptor in a pathway that is known to positively regulate Wg transcription at the dorsal/ventral boundary (Diaz-Benjumea and Cohen 1995; Rulifson and Blair 1995; Doherty et al. 1996; Rubinfeld et al. 1996) is required for Wg derepression (Rulifson et al. 1996). Although *Notch* could be acting in parallel with *dsh* in this process, it is interesting to note that Dsh protein has been shown to bind to and inhibit Notch activity when overexpressed in the wing (Axelrod et al. 1996). This makes for a model in which *wg* represses its own transcription by inhibiting *Notch* activity through *dsh*. Further studies of this side branch of the Wg pathway are needed, and it will be interesting to see whether this interaction is seen in other tissues.

Do Wnt proteins affect cell adhesion directly?

Before it was recognized that Arm/β-catenin forms a complex with Tcf proteins in the nucleus, a direct effect on cell adhesion was often suggested for Wnt proteins because of Arm/β-catenin's ability to bind cadherins.

Cell lines transfected with Wnt proteins can have altered cell adhesion properties (Bradley et al. 1993; Hinck et al. 1994), but at least in one case, this was shown to be due to increased transcription of cadherin (Yanagawa et al. 1997). Overexpression experiments in flies with wild-type and a dominant-negative version of cadherin are consistent with the notion that regulation of cell adhesion is not the major readout of Wg signaling (Sanson et al. 1996). Overexpression of β-catenin mutant proteins that cannot bind cadherin can still induce a secondary axis in *Xenopus* embryos (Funayama et al. 1995).

In *Drosophila*, a thorough structure–function analysis of *arm* demonstrated that embryos mutant for an allele of *arm* that is wild type for adhesion function but appears null for Wg signaling (it can bind Tcf but cannot activate transcription) could be rescued by an *arm* transgene that is defective in adhesion function (Orsulic and Peifer 1996). Thus *arm*'s two functions can be completely separated. A more subtle role for nontranscriptional changes in cellular adhesion during Wnt signaling cannot be ruled out, but rigorously demonstrating the existence of such a role in a living organism will be difficult.

Future directions

The plethora of Wnt proteins playing important roles in many developmental systems and in human disease has attracted increasing numbers of researchers, dramatically accelerating progress toward understanding Wnt signaling. Still, at every level of the pathway, major questions remain. How do the Fz receptors work? What is Dsh doing to transduce the signal? What is the relationship between APC and Wnt signaling? How does Arm/β-catenin get into the nucleus? Identification of factors that interact with the identified components of the pathway will undoubtedly lead to new discoveries and insights in cell culture systems and organisms such as *Xenopus*. In addition, there is still plenty of genetics left to do in flies and worms.

Despite the pioneering role of *Drosophila* in elucidating the first outline of a Wnt signaling pathway, it is important to realize that despite the intensive effort already made, the genetics of *wg* signaling is still in its infancy. Although the *Drosophila* genome has been nearly saturated for zygotic mutants specifically affecting segmentation, most components of Wg signaling are expressed both maternally and zygotically. Only one chromosome, the X, has been searched extensively for such genes (Perrimon et al. 1989). Such screens are now being performed on the autosomes (Perrimon et al. 1996), which identified the proteoglycan synthesis mutants described earlier. Modifier screens in the embryo and eye turned up the *DTcf* mutants (Brunner et al. 1997), and another eye modifier screen showed a gene named *eyelid*, which encodes a Bright transcription family member (Treisman et al. 1997). This gene acts antagonistically towards *wg*, its phenotype suggesting that it acts in parallel to the pathway to restrict Wg target gene expression. The next few years will see many more interesting fly mutations affecting Wg signaling, and new mutations in the worm will surely be found. These genes will almost certainly have vertebrate counterparts acting in similar ways. Likewise, results obtained in vertebrates will influence the work done in model systems. Thus, the widespread occurrence of Wnt signaling in animals guarantees that the rapid increase in the understanding of the pathway will continue.

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Wingless Repression of *Drosophila frizzled 2* Expression Shapes the Wingless Morphogen Gradient in the Wing

Kenneth M. Cadigan, Matthew P. Fish, Eric J. Rulifson, and Roel Nusse*
Howard Hughes Medical Institute
Department of Developmental Biology
Stanford University Medical Center
Stanford, California 94305

Summary

In *Drosophila* wing imaginal discs, the Wingless (Wg) protein acts as a morphogen, emanating from the dorsal/ventral (D/V) boundary of the disc to directly define cell identities along the D/V axis at short and long range. Here, we show that high levels of a Wg receptor, *Drosophila* frizzled 2 (Dfz2), stabilize Wg, allowing it to reach cells far from its site of synthesis. Wg signaling represses *Dfz2* expression, creating a gradient of decreasing Wg stability moving toward the D/V boundary. This repression of *Dfz2* is crucial for the normal shape of Wg morphogen gradient as well as the response of cells to the Wg signal. In contrast to other ligand–receptor relationships where the receptor limits diffusion of the ligand, Dfz2 broadens the range of Wg action by protecting it from degradation.

Introduction

Morphogens are defined as localized factors that can diffuse and, in a concentration-dependent manner, directly specify different cellular identities among a group of cells. This concept has long held attraction for developmental biologists because it offers an economical explanation for complex pattern formation (reviewed in Slack, 1987b; Lawrence and Struhl, 1996; Neumann and Cohen, 1997a). Because sequential signaling cascades are not easily ruled out, the existence of morphogens in multicellular developmental fields has been difficult to prove (Neumann and Cohen, 1997a). At present, one of the best examples of a morphogen is Wingless (Wg), a *Drosophila melanogaster* member of the Wnt family of proteins (Zecca et al., 1996; Neumann and Cohen, 1997b).

Wg is required for many cell fate decisions during embryonic and larval development (reviewed in Klingensmith and Nusse, 1994), and it has been suggested to act as a morphogen in several contexts (Bejsovec and Martinez Arias, 1991; Struhl and Basler, 1993; Hoppler and Bienz, 1995; Lawrence et al., 1996). However, the best documentation of such a role is in wing imaginal discs, the progenitors of adult wings (Zecca et al., 1996; Neumann and Cohen, 1997b). In this tissue, *wg* is expressed in a narrow stripe of four to five cells straddling the dorsal/ventral (D/V) boundary of the disc (Baker, 1988; Williams et al., 1993; Couso et al., 1994). Wg activates the expression of several genes adjacent to the stripe, including the proneural gene *achaete* (*ac*) (Phillips and Whittle, 1993; Couso et al., 1994). In addition to these short-range targets of Wg signaling, *Distal-less* (*Dll*) is expressed in a Wg-dependent manner in a much wider domain centering on the D/V stripe. A series of elegant experiments by Zecca et al. (1996) and Neumann and Cohen (1997b) using loss- and gain-of-function clonal analysis of Wg signaling components support a model where Wg directly acts at a distance to activate *Dll* transcription. Their data are consistent with a morphogen mechanism where a high level of Wg signaling is required to activate short-range targets like *ac*, whereas a lower threshold is needed for long-range targets such as *Dll*. They conclude that Wg can act up to at least 20 cell diameters away from its site of synthesis.

We have found that the protein distribution of Wg in the wing disc supports the morphogen model. It has previously been reported that Wg is present at highest levels in the cells of the D/V stripe expressing *wg* RNA. The concentration of Wg rapidly decreases moving away from the stripe, and after a few cell diameters it is found at low levels (Couso et al., 1994; Neumann and Cohen, 1997a). We show here that these low levels extend up to 25 cell diameters away from the D/V boundary, consistent with the genetic estimate of the range of Wg action. Our data indicate that the Wg morphogen gradient is biphasic, initially displaying a steep decreasing slope followed by a more gradual decline.

What factors dictate the distribution of the Wg morphogen? In simple local source-dispersed sink models (Slack, 1987a), the shape of a morphogen gradient is a function of the rate of diffusion of the morphogen away from the site of synthesis and its rate of degradation. However, modeling assuming a constant diffusion and degradation rate across the D/V axis cannot explain the observed biphasic nature of the Wg gradient. One must postulate an initial slow rate of diffusion and/or rapid turnover of the protein close to the D/V boundary, with greater diffusion rates and/or slower degradation further from the source of Wg. We present evidence that the rate of Wg turnover does decrease at a distance from the boundary. This modulated degradation is mediated by Wg-dependent regulation of the expression of *Drosophila frizzled 2* (*Dfz2*), a member of the Frizzled family of cell surface receptors.

Dfz2 has been shown to act as a Wg receptor in cultured cells (Bhanot et al., 1996). We show here that *Dfz2* expression in the wing is inhibited by Wg signaling. Uncoupling of *Dfz2* repression by Wg results in elevated levels of Wg far from its site of synthesis due to increased stability, as well as a dramatic expansion of Wg target gene expression. Thus, Wg regulation of *Dfz2* creates a negative feedback loop in which newly secreted Wg is very unstable until it moves away from the D/V boundary to cells expressing a higher level of *Dfz2*. We find no evidence for Dfz2 influencing the rate of Wg diffusion and propose that the differential stability of Wg explains both the initial steep slope and the subsequent plateau of the Wg gradient. We conclude that the interaction between Wg and Dfz2 plays a crucial role in shaping the Wg morphogen gradient and determining the response of cells to the Wg signal.

*To whom correspondence should be addressed.

Results

Wg Signaling Inhibits *Dfz2* Expression

Dfz2 has previously been shown to bind and transduce the Wg signal in cell culture (Bhanot et al., 1996). To examine its role in vivo, we examined its expression pattern in the developing wing. In the wing pouch, the region of the disc destined to become wing blade, *Dfz2* is expressed in an inverse pattern to that of Wg, with the lowest levels found at the D/V boundary (Figures 1A and 1B). This pattern is Wg-dependent, since *Dfz2* expression near the D/V stripe is derepressed when Wg activity is blocked for 24 hr in wg^{ts} discs (Figure 1D) compared to wg^{ts} discs at the permissive temperature (Figure 1C). To extend these findings, we utilized the Gal4/UAS system of Brand and Perrimon (1993) to express deleted versions of two Wg signaling components, armadillo and dTCF, which constitutively activate (arm^{act}; Pai et al., 1997) or inhibit ($dTCF^{DN}$; van de Wetering et al., 1997) Wg signaling. Expression of arm^{act} throughout the wing pouch represses *Dfz2* expression (Figure 1E), while expression of $dTCF^{DN}$ in a Patched (Ptc) pattern (i.e., a stripe that runs perpendicular to the D/V *wg* stripe at the anterior/posterior boundary) leads to derepression of *Dfz2* within the Ptc domain (Figure 1F). Thus, Wg signaling is responsible for the lower expression of *Dfz2* near the D/V boundary.

Uniform Expression of *Dfz2* in the Wing Pouch Expands Wg Target Gene Expression

To test whether *wg*-dependent repression of *Dfz2* expression is important for normal wing development, we misexpressed *Dfz2* using UAS-Dfz2 lines crossed to various Gal4 drivers (Brand and Perrimon, 1993). Figure 2 shows the consequences of placing *Dfz2* under the control of 1J3-Gal4. We found that this Gal4 driver is expressed in every cell in the wing pouch except those at the D/V boundary (Figure 2C), and 1J3-Gal4/UAS-Dfz2 (1J3/Dfz2) expression (Figure 2E) overwhelms the endogenous graded *Dfz2* pattern (Figure 2D).

1J3-Gal4 is active in many larval tissues (data not shown), and 1J3/Dfz2 flies are usually pupal lethal. However, adults can be obtained by reducing Gal4 activity through lower rearing temperatures or by using UAS-Dfz2 lines with weaker expression levels due to position effect variation. In either case, all surviving animals have ectopic bristles on their wing blades (Figures 2A and 2B). These sensory organs are normally only found at the wing margin, the adult structure corresponding to the D/V boundary, and depend on Wg activity for their formation (Phillips and Whittle, 1993; Couso et al., 1994). The ectopic bristles in the anterior compartment were almost always of the slender or chemosensory type (Figure 3B), though an occasional stout bristle is also observed (data not shown). In the posterior compartment, the extra bristles are similar to the noninnervated ones found at the posterior margin. This "hairy wing" phenotype was also seen with other Gal4 drivers, such as 71B and 69B, which are also expressed in the developing wing blade (data not shown).

The slender and chemosensory bristle cell fates are determined during the third larval instar by proneural genes such as *ac*, whose expression is *wg*-dependent (Phillips and Whittle, 1993; Couso et al., 1994). *ac* is initially expressed at mid-third instar in the anterior compartment in a stripe on each side of the D/V boundary. The cells destined to become bristle precursors gradually accumulate Ac to higher levels than their neighbors (Campuzano and Modolell, 1992; see Figure 2F). Consistent with the hairy wing phenotype, 1J3/Dfz2 discs have

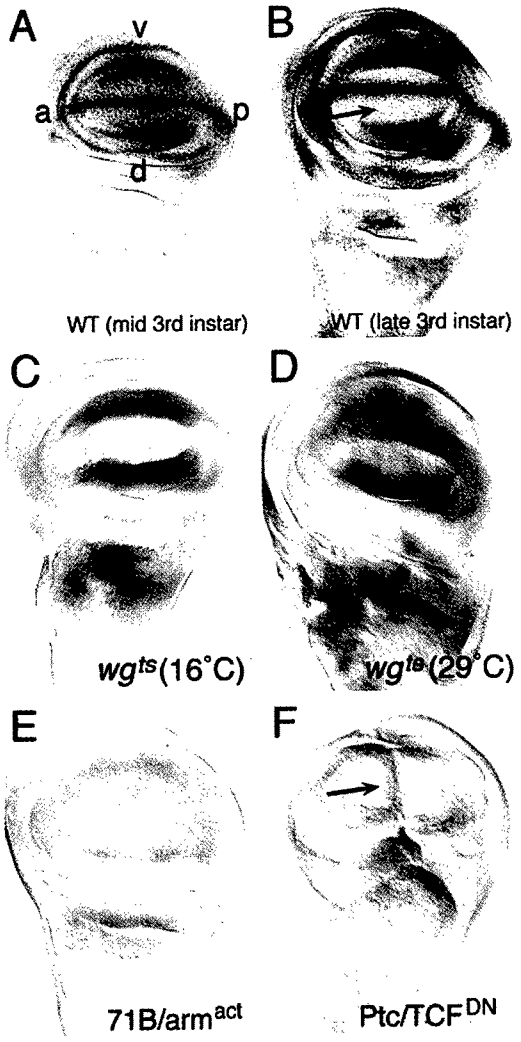

Figure 1. Wg Signaling Represses *Dfz2* Transcript in Wing Imaginal Discs

Wing discs were stained for Wg (brown) and *Dfz2* mRNA (purple; A and B) or *Dfz2* mRNA alone (C–F). In wild-type discs, the *Dfz2* pattern evolves from mid (A) to late (B) third larval instar, with *Dfz2* levels lowest close to high levels of Wg. (A) shows the anterior (a), posterior (p), dorsal (d), and ventral (v) orientation of the discs. Note that *Dfz2* transcripts are low but not absent close to the Wg D/V stripe (arrow). This repression of *Dfz2* expression is Wg-dependent, since it is abolished in wg^{ts} discs reared at the restrictive temperature (29°C) for 24 hr prior to fixation (D). Note that wg^{ts} discs reared at 16°C (C) had a *Dfz2* pattern similar to wild-type. 71B/arm^{act} discs, which constitutively activate Wg signaling throughout the wing pouch, result in much lower *Dfz2* levels (E). In Ptc/$dTCF^{DN}$ discs (F), where Wg signaling is blocked in a narrow stripe perpendicular to the D/V boundary, derepressed levels of *Dfz2* are observed where the Ptc domain is predicted to be (arrow).

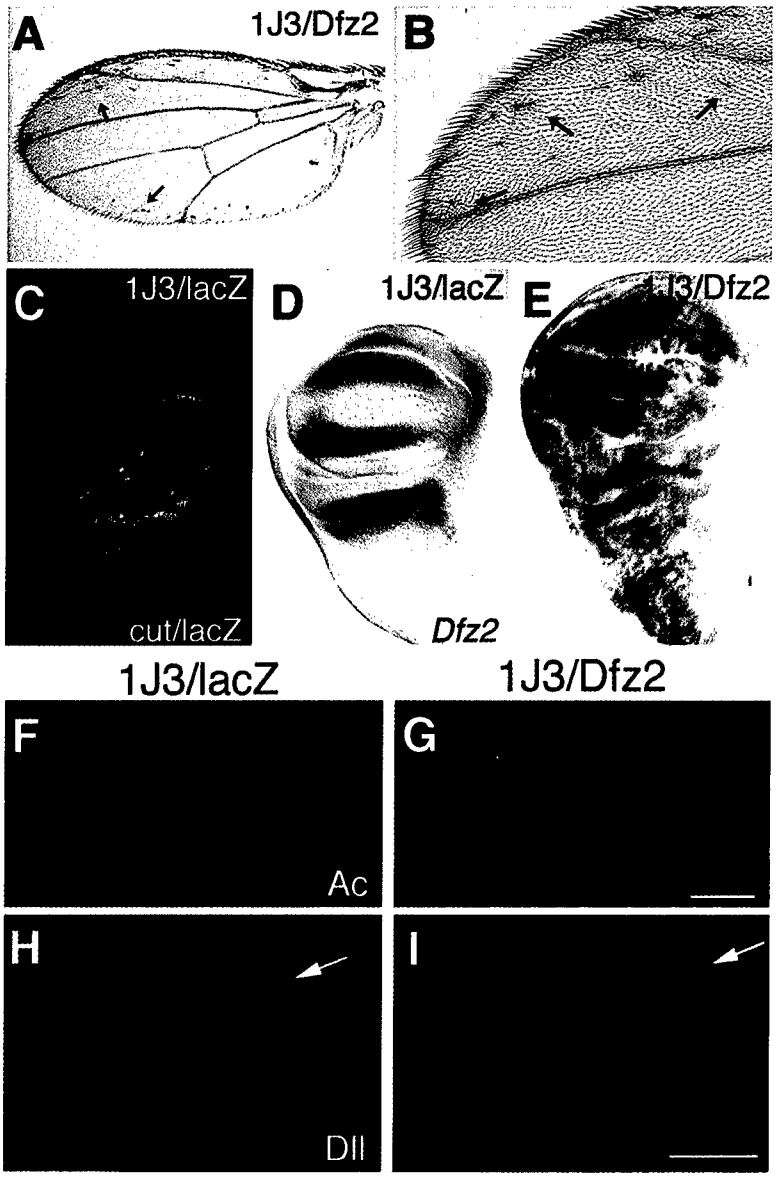

Figure 2. Ectopic Expression of *Dfz2* throughout the Wing Disc Causes an Expansion of Wg Target Gene Expression, Resulting in "Hairy" Wings

1J3/Dfz2 animals are pupal lethal when reared at 29°C, but at lower temperatures some escapers with wings containing ectopic bristles (arrows) are obtained (A and B). The 1J3-Gal4 enhancer trap is active at high levels throughout the wing except in the *wg* producing cells at the D/V boundary, as demonstrated by 1J3/lacZ discs stained for lacZ (red) and cut (green), a marker for the D/V boundary (C). At 29°C, 1J3/Dfz2 discs stained for *Dfz2* transcripts revealed that the endogenous spatial pattern of 1J3/lacZ controls (D) is overwhelmed by the transgenic *Dfz2* (E). (F)–(I) show that discs immunostained for the Wg targets Ac (green; F–G) and *Dll* (red; H–I) have significantly expanded expression domains in 1J3/Dfz2 (G and I) compared to 1J3/lacZ controls (F and H; compare arrows in [H] and [I]). Note that, in addition to fully penetrant elevated *Dll* expression away from the D/V boundary, *Dll* levels near the source of Wg are higher in (I) compared to (H). This effect was seen approximately half of the time. Bar in (G), 25 μM; in (I), 100 μM.

a dramatic increase in cells expressing high levels of Ac (Figure 2G). These cells are found at a greater distance from the D/V stripe than in controls and presumably cause the ectopic bristles seen in adult wings.

In the morphogen model of Wg action in the wing blade (Zecca et al., 1996; Neumann and Cohen, 1997b), *ac* is an example of a short-range target, requiring high levels of Wg signaling to be expressed. As was found for *ac*, the expression domain of another short-range target, *Delta (Dl)*, normally expressed in a narrow stripe on either side of the *wg* stripe (Micchelli et al., 1997), is much broader in 1J3/Dfz2 wing discs (data not shown). The model also states that Wg acts directly on long-range targets, such as *Dll*, which require less Wg signaling for activation and thus are normally expressed in wider domains centered on the D/V boundary (Zecca et al., 1996; Neumann and Cohen, 1997b). *Dll* is expressed at highest levels close to the Wg stripe and then at progressively lower levels at further distances (Figures 2H and 3C). In 1J3/Dfz2 discs, the higher expression levels of *Dll* are seen much further from the stripe (Figure 2I) than in controls (Figure 2H; see arrows). Thus, misexpression of *Dfz2* at high levels throughout the wing pouch expands the domains of both short- and long-range Wg targets.

The Dfz2 Phenotype Is Wg-Dependent, and a Truncated Form of Dfz2 Blocks Wg Signaling

The increased activation of Wg targets by misexpression of *Dfz2* could be due to a heightened response of the cells to the Wg signal, or a constitutive activation of the signaling pathway. To address this, we examined the effect of *Dfz2* misexpression in discs from wg^{ts} mutants reared at the restrictive temperature. Both Ac and *Dll* expression were dramatically reduced in these discs (Figure 3B) to levels seen in wg^{ts} discs grown under the same conditions. This indicates that the primary effect

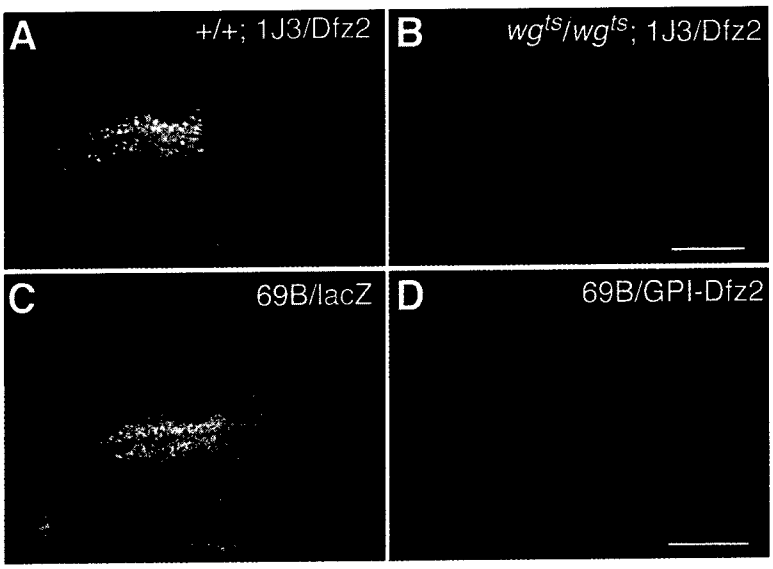

Figure 3. Wg Signaling Is Activated by Full-Length Dfz2 and Blocked by a Truncated Dfz2 Containing only the Extracellular Domain

All discs were stained for Ac (green) and *Dll* protein (red; overlap is yellow). The expanded expression of Ac and *Dll* observed in 1J3/Dfz2 discs is Wg-dependent, since their levels are dramatically decreased in 1J3/Dfz2 discs in a wg^{ts} background reared at the restrictive temperature of 29°C (n = 6) for 24 hr prior to fixation (B) compared to 1J3/Dfz2 discs reared the same way (A). In discs expressing GPI-Dfz2 throughout the wing pouch using the 69B-Gal4 driver, Ac and *Dll* expression are also dramatically reduced (D) compared to 69B-Gal4/UAS-lacZ discs (C) where their patterns are identical to wild-type. The remaining Ac expression in (B) and (D) is not Wg-dependent (Phillips and Whittle, 1993). Bars, 100 μM.

of *Dfz2* misexpression is to potentiate the ability of Wg to signal to target cells.

The 1J3/Dfz2 experiments suggest that Dfz2 can transduce the Wg signal in the wing. Presumably, as has been shown in cell culture, this occurs through direct binding (Bhanot et al., 1996). To examine this in more detail, we expressed in flies an altered *Dfz2* cDNA predicted to encode the extracellular domain anchored to the cell surface via a glycerol-phosphatidyl inositol linkage. This truncated protein (GPI-Dfz2) binds Wg in cell culture (Bhanot et al., 1996) but should not be able to transduce the signal to intracellular targets, since it lacks the seven transmembrane and intracellular domains. Therefore, if Dfz2 and Wg can interact in vivo, GPI-Dfz2 should block Wg signaling by binding the protein nonproductively.

Expression of GPI-Dfz2 in the wing pouch does abolish the expression of the Wg targets *ac* and *Dll* (Figure 3D) and causes severe notching of the wings in adults (data not shown). Experiments in the embryo and eye

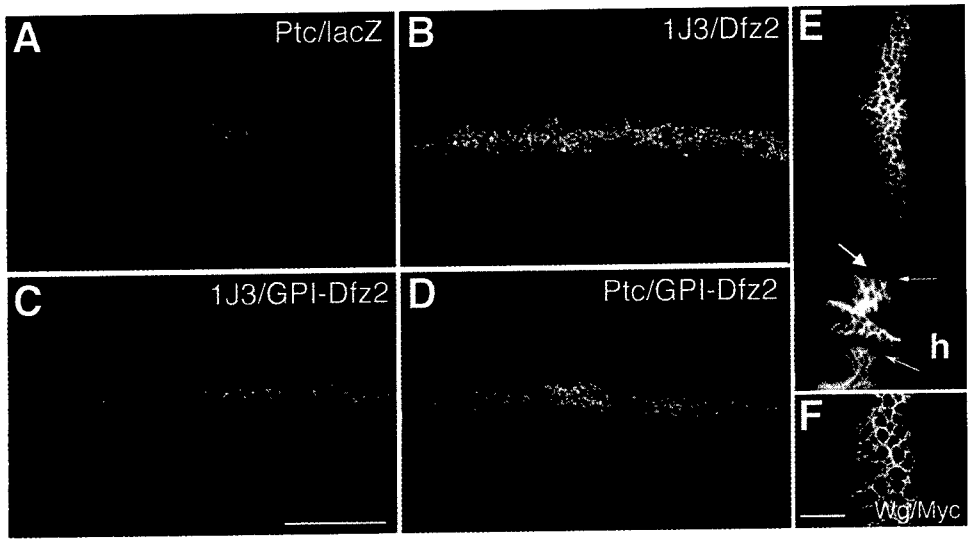

Figure 4. Ectopic Expression of Dfz2 or GPI-Dfz2 Leads to Posttranscriptional Accumulation of Wg

Wing discs were stained for Wg protein (green) and *wg* RNA (red; overlap is yellow). Ptc/lacZ (A) discs display a wild-type pattern with protein levels dropping off rapidly outside the RNA expression domain but with a punctate signal extending a considerable distance. Identical results were obtained with 1J3/lacZ (data not shown). 1J3/Dfz2 discs have high levels of Wg several cell diameters away from the RNA stripe (B). The effect is even more dramatic in 1J3/GPI-Dfz2 discs, where Wg is found almost throughout the presumptive wing pouch (C). Ptc/GPI-Dfz2 discs have a remarkable posttranscriptional elevation of Wg in the Ptc domain (D). This Wg colocalizes with GPI-Dfz2 (the GPI-Dfz2 protein contains a myc epitope and was detected with a monoclonal anti-myc antibody) at the cell surface (F). (E) shows Myc (red) and Wg (green) costaining. Wg from the dorsal hinge region (h) abruptly stops at the fold between the hinge and wing blade primordia (top slender arrow). The cells in the fold (which lies below the focal plane; fat arrow) contain similar levels of Myc staining as seen along the rest of the A/P border. A similar block in Wg movement is also seen at the more proximal fold (lower slender arrow) and the ventral hinge region (data not shown). (B)–(F) are a more apical optic section than (A), so the endogenous punctate pattern seen in (A) is not visible. Bar in (D), 50 μM; bar in (F), 10 μM.

indicate that GPI-Dfz2 efficiently blocks Wg signaling in these tissues as well (Cadigan et al., unpublished data). These data are consistent with the hypothesis that Dfz2 is a physiologically relevant Wg receptor.

Misexpression of Dfz2 Alters Wg Distribution by Increasing Its Stability

Wg is normally found at high levels in the cells expressing *wg* RNA but drops off sharply moving away from the stripe (Couso et al., 1994). Previously, it has been reported that Wg is undetectable more than 10 cell diameters from the D/V boundary (Neumann and Cohen, 1997a). Using an affinity-purified Wg antibody (Bhanot et al., 1996), we find that low levels of Wg are still detected up to 25 cell diameters away from the site of secretion (Figures 4A and 6F). This Wg signal is punctate and favors the apical portion of the epithelium. It is not seen in wg^{ts} discs grown at the restrictive temperature (Figure 6G), indicating that it is due to Wg and not a cross-reaction artifact. Thus, the physical distribution of Wg is consistent with the genetic evidence that it can directly affect gene expression over long distances (Zecca et al., 1996; Neumann and Cohen, 1997b).

Misexpression of *Dfz2* or GPI-Dfz2 causes a dramatic posttranscriptional spread of Wg, with 1J3/Dfz2 discs having high levels of Wg several cell diameters away from the RNA stripe (Figure 4B; the RNA stripe is somewhat wider than normal for reasons that are unclear). The effect is more pronounced with GPI-Dfz2 (Figure 4C), and Wg can be found at moderately high levels 20–25 cell diameters away in Ptc-Gal4/UAS-GPI-Dfz2 (Ptc/GPI-Dfz2) discs (Figure 4D). We are unable to detect Dfz2 adequately with immunostaining, but GPI-Dfz2 contains a myc epitope, allowing its detection. Figure 4F shows that in Ptc/GPI-Dfz2 discs, GPI-Dfz2 and Wg colocalize at the cell surface.

We used Western blot analysis to measure the apparent accumulation of Wg in these wing discs (Figure 5). 1J3/Dfz2 and 1J3/GPI-Dfz2 discs had 1.7× and 5.6× more Wg than controls, respectively (in a separate experiment, the differences were 2.1× and 2.7×, respectively). Ptc/GPI-Dfz2 also had 2.2× its control. The differences roughly reflect the increases in immunostaining shown in Figure 4. While we cannot rule out that some of the increased Wg staining is due to better fixation of Wg, the Western analysis indicates that discs expressing *Dfz2* or GPI-Dfz2 do contain more Wg than normal.

When direct comparisons with the same Gal4 driver could be made, we always saw a greater accumulation of Wg with GPI-Dfz2 compared to Dfz2 (Figures 4B, 4C, and 5; Figure 6C legend). This could simply be due to a higher amount of truncated receptor present or caused by the inability of GPI-Dfz2 to internalize Wg after binding.

In early third larval instar, about 48 hr prior to the stage shown throughout this paper, *wg* is expressed throughout the entire wing pouch (Phillips and Whittle, 1993; Williams et al., 1993). Thus, the accumulation of Wg could be due to a posttranscriptional stabilization of this earlier pattern, rather than stabilization of Wg traveling from the D/V stripe at late third instar. To address this, we induced clones of cells homozygous for

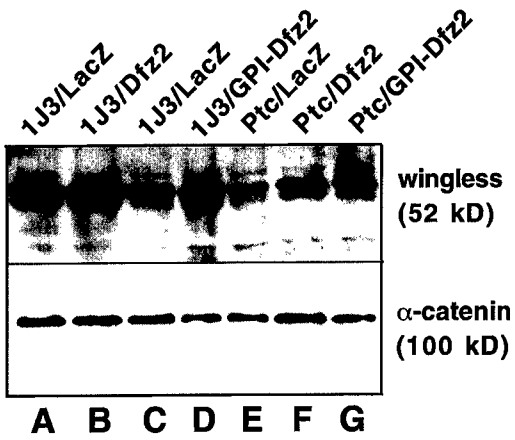

Figure 5. Western Blot Analysis Demonstrates Elevated Levels of Wg in Discs Ectopically Expressing Dfz2 or GPI-Dfz2

Cell extracts of approximately 15 imaginal discs were subjected to Western blot analysis with monoclonal antibodies against Wg (top panel) or α-catenin (bottom panel) as a loading control. Extracts were as followed: lane A, 1J3/lacZ and lane B, 1J3/Dfz2, reared continuously at 29°C; lane C, 1J3/lacZ; lane D, 1J3/GPI-Dfz2; lane E, Ptc/lacZ; lane F, Ptc/Dfz2; lane G, Ptc/GPI-Dfz2, all reared at 18°C and then 29°C for 24 hr before dissection. The signal was quantitated by densitometry, and the ratio of Wg/α-catenin signal (in arbitrary units with the control lanes A, C, and E normalized to 1.0) were as follows: lane B, 1.7; lane D, 5.6; lane F, 1.3, and lane G, 2.2. The control units in lanes C and E are 52% and 44% of that in lane A.

a Wg null mutation (van den Heuvel et al., 1993) in a 1J3 or Ptc/GPI-Dfz2 background. As seen in Figures 6A and 6B, large clones lacking the ability to make Wg still accumulated the protein to levels seen in adjacent Wg-producing tissue. This indicates that the Wg in the clones must have diffused from another location. Evidence that this source is the D/V stripe comes from the data in Figures 6C–6E. Random clones of cells expressing Gal4 under the control of an actin promoter (Pignoni and Zipursky, 1997) were made in a UAS-Dfz2 background. When the clones were induced 24 hr prior to fixation, a time when the overall expression pattern of *wg* has faded and the D/V stripe has emerged (Rulifson et al., 1996) (Figure 2A), *Dfz2*-expressing cells up to 12 cell diameters away from the stripe accumulate Wg (Figure 6C). Induction of clones 3.5 days before fixation did not alter the distance from the stripe at which elevated levels of Wg was observed (Figure 6E), suggesting that the earlier expression pattern of *wg* had not significantly contributed to the effect on Wg seen at the late stage. Taken together, these data suggest that the graded accumulation of Wg seen on the *Dfz2* or GPI-Dfz2–overexpressing cells was derived from the D/V stripe.

The immunostaining and Western analysis indicate that high levels of Dfz2 or GPI-Dfz2 stabilize Wg. We sought to determine whether the accumulation of the protein so far from its site of synthesis is also due to increased diffusion or transport. At a given distance from the source of Wg at the D/V boundary, cells in clones expressing *Dfz2* have the same levels of elevated Wg (arrows in Figures 6D and 6E) whether or not the clone is in contact with D/V stripe. Since high levels of

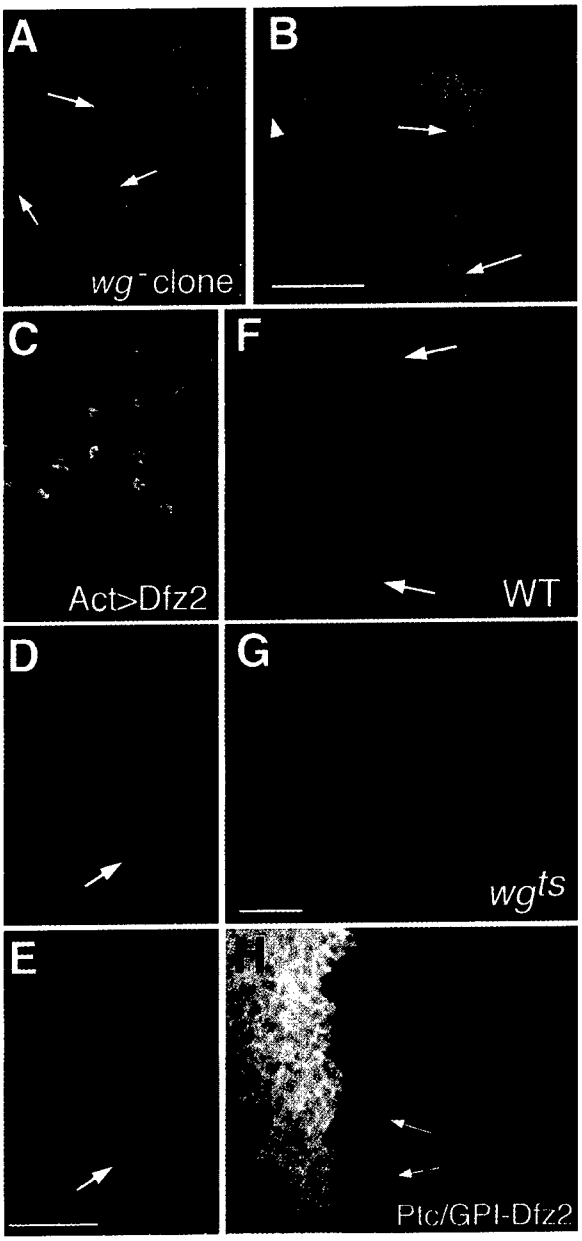

Figure 6. The Accumulated Wg Is Derived from the D/V Stripe and Travels by a Mechanism that Appears Not to Be Dfz2-Dependent

Discs were stained for Wg (green) and where indicated, lacZ (red). (A) and (B) show *wg* mutant clones, marked by the absence of lacZ (borders of clones indicated in white) in a 1J3/GPI-Dfz2 (A) and Ptc/GPI-Dfz2 (B) disc. Clones at the D/V boundary abolish Wg expression (arrowhead in [B]) but no effect on Wg levels were observed in any of the 12 large clones outside the *wg* RNA-expressing domain examined (arrows).

(C) shows that random clones (marked by the presence of lacZ) expressing Dfz2 from the actin 5c promoter accumulate Wg to high levels up to 8 cells from the D/V stripe and higher than normal levels until 12 cell diameters away. The clones were induced at 24 hr prior to fixation and did not contain *wg* RNA (data not shown). Clones expressing GPI-Dfz2 accumulated high levels of Wg to 12 cell diameters away from the stripe (data not shown).

(D) and (E) show larger actin/Dfz2 clones (borders of clones indicated in white) induced at first larval instar. The arrows indicate approximately 12 cell diameters from the *wg* RNA expression domain, and actin/Dfz2 cells at this distance have similar levels of Wg (note that the gain of the confocal was set higher for [D] than [E] to more clearly show the ectopic Wg in [D]). Five clones of each type were examined with similar results. Clones of GPI-Dfz2 induced at this early time prevented wing blade formation. (F) and (G) show Wg patterns in wild-type (F) and wg^{ts} (G) discs reared at the restrictive temperatures for 12 hr prior to fixation. The punctate Wg signal seen at a distance from the site of synthesis in wild-type (arrows) is absent in the wg^{ts} disc (the RNA stripe in wg^{ts} discs is approximately twice as wide as controls due to Wg negative regulation of its transcription; see Rulifson et al. 1996).

(H) shows a Ptc-Gal4/+; UAS-GPI-Dfz2/UAS-lacZ disc. Note that there is higher than normal Wg staining in the cells adjacent to the posterior border of the Ptc domain (marked by lacZ staining), indicating that Wg is able to diffuse away from the GPI-Dfz2–expressing cells in which it accumulates. Bar in (B), 100 μM; bars in (E) and (G), 25 μM.

Dfz2 near the Wg source are not required for Wg to accumulate many cell diameters away, this argues that the increased *Dfz2* expression is not enhancing movement of Wg.

While Dfz2 does not appear to facilitate the diffusion of Wg directly, its ability to protect Wg from degradation can indirectly promote the movement of Wg. This is demonstrated in Figure 6H, where Wg protein is seen at higher than normal levels in the proximal region of Ptc/GPI-Dfz2 discs, adjacent to the Ptc expression domain. The data strongly suggest that the Wg is derived from the GPI-Dfz2–expressing cells. Presumably, some Wg can dissociate from GPI-Dfz2 and diffuse to neighboring cells.

Uncoupling the Endogenous *Dfz2* Gene from Wg Regulation Alters Wg Distribution

The data presented thus far are based on expression of an artificial protein (GPI-Dfz2) and heterologous promoters that express *Dfz2* at higher than normal levels. Therefore, we manipulated endogenous *Dfz2* levels by activating or inhibiting Wg signaling and determined the effect on Wg levels. Expression of $dTCF^{DN}$, which blocks Wg signaling (van de Wetering et al., 1997), in the posterior compartment of wing discs results in derepressed levels of *Dfz2* transcripts (Figure 7A) and accumulation of Wg outside the RNA expression domain (Figure 7B). Clones mutant for *dishevelled (dsh)* activity, which lack Wg signaling (Couso et al., 1994; Klingensmith et al., 1994), also have a similar accumulation of Wg (Figure 7C). Conversely, clones lacking *zeste white 3* (*zw3*; also known as *shaggy*), which constitutively activate Wg signaling (Siegfried et al., 1992; Blair, 1994) and are predicted to have repressed *Dfz2* levels, have less Wg inside them compared to surrounding tissue (Figure 7D). These results show that Wg signaling has a negative effect on the accumulation of Wg, which can be explained by its ability to inhibit *Dfz2* expression.

Discussion

There is abundant evidence implicating Frizzleds as Wnt receptors (reviewed in Cadigan and Nusse, 1997), and, in cell culture, the Frizzled family member Dfz2 can function as a Wg receptor (Bhanot et al., 1996). In flies, misexpression of full-length Dfz2 results in stabilization of Wg and the expansion of Wg target gene expression

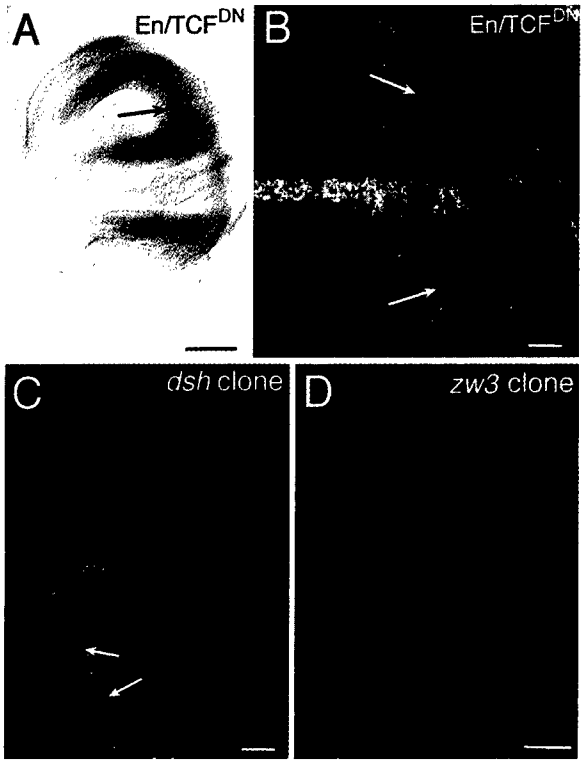

Figure 7. Wg-Dependent Regulation of *Dfz2* Is Required for Normal Wg Distribution

Wing discs from En/dTCF^{DN} larvae were stained for *Dfz2* RNA (A) or Wg (green) and *wg* RNA (red; [B]). The cells expressing the dTCF^{DN} in (B) were also marked with lacZ (data not shown) and are to the right of the white line. The Wg stripe is dramatically wider in the dTCF^{DN} cells and is found beyond the RNA stripe (arrows). These cells have levels of Dfz2 transcripts only normally seen at a distance from the D/V boundary (A).

(C) shows a dsh mutant clone (marked by the absence of myc staining indicated by the white lines). Wg expands a considerable distance in the clone, presumably due to elevated *Dfz2* expression.

(D) shows that a *zw3* clone (marked by the absence of lacZ indicated by the white lines), which is predicted to repress *Dfz2* expression, has a lower amount of Wg compared to cells outside the clone. Nine other clones of similar size also showed this effect. Bar in (A), 100 μM; other bars, 10 μM.

in a Wg-dependent manner. Moreover, the extracellular domain of Dfz2 efficiently blocks Wg signaling and colocalizes at the membrane with stabilized Wg, which strongly suggests in vivo binding. The effects of Dfz2 on Wg distribution and target gene expression do not occur with the closely related protein, Frizzled (Cadigan et al., unpublished data), indicating specificity of Dfz2 for Wg signaling. While these results do not conclusively demonstrate that Dfz2 is a physiological receptor for Wg, they are consistent with that hypothesis.

Wg-Mediated Repression of *Dfz2* Expression Shapes the Wg Morphogen Gradient

A strong case has been made that Wg secreted at the D/V boundary of the presumptive wing blade in wing imaginal discs operates as a morphogen (Zecca et al., 1996; Neumann and Cohen, 1997b), with short-range targets including *ac* and *Dl*, and long-range targets such as *Dll*. In this paper, we demonstrate that the expression of *Dfz2* is repressed by Wg. This regulation is important for normal wing development, since overriding it with exogenous Dfz2 causes a marked expansion of the domains of both short- and long-range Wg targets (Figure 2). This effect is strictly dependent on *wg* activity (Figure 3B), indicating that misexpression of Dfz2 increased the level of signaling by the Wg morphogen.

This increase in target gene activation may in part be caused by the dramatic effect that Dfz2 misexpression has on Wg distribution. Uniformly high levels of Dfz2 cause an increase in the amount of Wg found in or on the Dfz2-expressing cells, even at considerable distances from the D/V stripe (Figures 4, 6, and 7). This accumulation as judged by immunostaining was confirmed by Western analysis (Figure 5). The effect is predominantly posttranscriptional (Figures 4 and 7B), as well as posttranslational, since the effect is still robust in clones of Dfz2-expressing cells not in contact with the cells expressing *wg* (Figure 6C). Therefore, Dfz2 protects Wg from degradation, presumably by direct binding. However, this binding is reversible, since higher than normal levels of Wg can be found adjacent to cells expressing the truncated form of Dfz2 (Figure 6H).

We propose that the ability of Dfz2 to stabilize Wg, combined with the Dfz2 expression pattern, plays a major role in shaping the Wg morphogen gradient. Wg concentration initially decreases rapidly moving away from the D/V boundary but then plateaus at a low level (Figures 4A and 6F). Our data support a model where Wg is normally able to travel up to 25 cell diameters away from its source, consistent with genetic data on the range of action of Wg (Zecca et al., 1996; Neumann and Cohen, 1997b). This diffusion/transport of Wg does not appear to be enhanced by increased Dfz2 levels (Figures 6C–6E). However, the distribution of Dfz2 creates a situation where Wg is unstable near the D/V boundary and more stable at further distances (Figure 8). High levels of Dfz2 near the boundary, through expression of a transgene (Figure 4B) or derepression of the endogenous *Dfz2* genes, stabilizes Wg so that elevated levels are observed. Repression of Dfz2 expression away from the boundary, via activation of the Wg signaling pathway, destabilizes Wg, resulting in lower levels found in these cells (Figure 7D). Thus, Dfz2-mediated stabilization of Wg can, in large part, explain the biphasic nature of the Wg morphogen gradient.

It is not clear whether the effect of Dfz2 misexpression on Wg target genes is due solely to the elevation of Wg levels available to the target cells. It is also possible that the cells are more responsive to the Wg signal due to the higher levels of a Wg receptor. However, the stainings of *Dfz2* transcripts shown in Figure 1 are nonquantitative, and we do not know how many molecules of *Dfz2* mRNA/cell are present, only that there are fewer near the D/V boundary than in more distal cells. There may already be a saturating level of Dfz2 receptor (in regard to transducing the Wg signal) even at the boundary, with Wg the factor in limiting supply. Whether the effects seen are primarily through regulating the responsiveness of cells to Wg or by modulating the amount of Wg ligand, it is clear that the *Dfz2* differential expression is required for the normal level of activation of Wg targets.

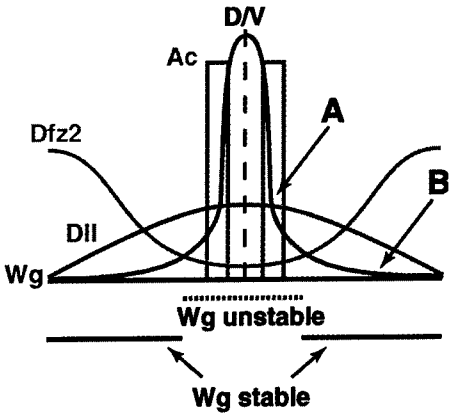

Figure 8. Wg Repression of *Dfz2* Helps to Shape the Wg Gradient and Response Profile

The D/V axis of the wing is shown schematically. The Wg gradient (blue line) has a very steep slope (A) moving away from the D/V boundary (the site of Wg synthesis) but then levels off after reaching a low level and extends for a considerable distance (B). The model states that Wg repression of *Dfz2* transcripts (pink line) near the D/V boundary creates an environment where Wg has a short half-life, leading to the steep slope. However, the small amount of Wg that reaches the distal regions containing higher *Dfz2* levels is stabilized, leveling off the slope. The steep slope restricts the expression domain of short-range targets such as Ac (green lines), and the distal stabilization allows Wg to activate long-range targets, such as *Dll* (red line). It is also possible that target gene domains are in part determined by a differential responsiveness of the cells to the Wg signal, due to the Dfz2 receptor gradient. The mechanism of Wg diffusion and/or transport is suggested not to be directly dependent on Dfz2.

Misexpression of Dfz2 in leg imaginal discs also alters Wg distribution and disrupts leg development (M. F., K. C., and R.N., unpublished observations). This suggests that regulated levels of Dfz2 may be generally important for Wg action, at least in imaginal discs.

Wg Diffusion/Transport

The mechanism of Wg transport in the wing blade remains unclear. However, we did find evidence for a block in Wg movement at the folds that separate the presumptive hinge region from that of the wing blade proper (Figure 4F). The nature of this barrier is not known. Its existence does explain why Wg expressed in the hinge region is not required for patterning the wing blade (Neumann and Cohen, 1996) and why the *Dll* promoter only responds to Wg from the D/V stripe (Zecca et al. 1996; Neumann and Cohen, 1997b).

Negative Feedback Circuits in Cell Signaling

Previously, it has been shown that Wg down-regulates its own transcription in the wing pouch to narrow the RNA stripe at the D/V boundary (Rulifson et al., 1996). In this paper, we present evidence for an additional negative feedback loop where high levels of Wg signaling repress *Dfz2* expression. Because Dfz2 is required for Wg stability, the protein has a short half-life near the D/V stripe. This causes a rapid decrease in Wg concentration a few cell diameters away from the boundary. This drop in ligand concentration, as well as a possible reduction in the responsiveness of cells to Wg due to low Dfz2 receptor levels, limits the distance at which Wg can activate short-range targets such as *ac* (Figure 8).

Though the biochemical mechanisms differ, the general strategy of negative feedback is a recurring theme for signaling molecules in *Drosophila* developmental fields. There are now several examples in flies where unrelated secreted signals induce the transcription of genes encoding proteins that inhibit the signaling pathway. These include *spitz* and *argos* (Golembo et al., 1996; Freeman, 1997), *decapentaplegic*, and *daughters against dpp* (Tsuneizumi et al., 1997), as well as *branchless* and *sprouty* (Hacohen et al. 1998). In addition, Hedgehog (Hh) activates the expression of the *patched (ptc)* gene in the wing disc (Capdevila et al., 1994; Tabata and Kornberg, 1994), and evidence has been presented that Ptc blocks the diffusion of Hh (Chen and Struhl, 1996). Patched proteins are thought to be receptors for Hedgehog family members (Marigo et al., 1996; Stone et al., 1996). Thus, Hh limits its range of action in the opposite way as Wg, by inducing rather than repressing a receptor's expression. As is the case in metabolic pathways, these feedback circuits may be a general principle in the homeostatic regulation of signaling levels during development.

Ligand Sequestration by Receptors

While the Wg/Dfz2 regulatory circuit joins the other recently described feedback loops to from a common theme, the fact that high levels of the Dfz2 receptor promote the spread of its ligand (through inhibition of Wg turnover) is contrary to other reported examples. In addition to the Hh/Ptc example described above (Chen and Struhl, 1996), there is also evidence that the Toll and Torso receptors act to sequester their respective ligands, Spätzle and Trunk, limiting signaling to the ventral or terminal portions of the early fly embryo (Stein et al., 1991; Casanova and Struhl, 1993). Also, the Let-23 receptor is thought to limit the diffusion of its ligand, Lin-3 in the worm (Hajnal et al., 1997). However, under no conditions of Dfz2 overexpression did we observe a block of Wg diffusion.

Why is Wg/Dfz2 different from the other cases? According to standard binding theory, the following three conditions would favor the majority of ligand binding to its receptor: high affinity binding, a high number of binding sites, and a concentration of ligand below the K_d (Hulme and Birdsall, 1992). For the specific ligand–receptor pairs under discussion, these factors have not been determined. However, of the ligands that are thought to be sequestered by their receptors, two (Lin-3 and Trunk) are predicted by sequence homology to require proteolytic processing for maturation (Hill and Sternberg, 1992; Casanova et al., 1995), and processing has been shown experimentally for the other two. The processed form of Spätzle is present at less than 1% of precursor levels (Morisato and Anderson, 1994), indicating that processing is rate limiting. The processing is more efficient for Hh but results in a mature form that is covalently linked to cholesterol (Porter et al., 1996b). This form of Hh is predominantly cell-associated, and this modification has been shown to be important in

limiting the range of Hh action (Porter et al., 1996a). Thus, it may not be surprising that these ligands are sequestered by their receptors, since the amount of freely diffusible mature ligand in the two cases examined is likely to be very low.

In contrast to the above examples, after entering the secretory pathway, Wg is not thought to require proteolytic processing, and significant amounts of biologically active Wg can be obtained in the media of *wg*-expressing cells (Van Leeuwen et al., 1994; Reichsman et al., 1996). Therefore, the concentration of extracellular Wg may simply be too high for sequestration to occur. Clearly, more ligand–receptor pairs must be examined and the existing ones studied in more biochemical detail to determine what factors govern the nature of their relationships.

Experimental Procedures

Fly Stocks

The pUAS-Dfz2 construct was created by cloning the 2.2 kB XhoI-SpeI fragment of the Dfz2 coding region from pMK33-Dfz2 (Bhanot et al. 1996) into pUAST (Brand and Perrimon, 1993). The 1.0 kB ClaI-ApaI fragment of pMRK5 (Bhanot et al., 1996) was used for the pUAS-GPI-Dfz2 derivative. Both constructs were introduced into w^{1118} hosts by P element–mediated transformation using standard methods (Rubin and Spradling, 1982). Thirteen UAS-Dfz2 and 11 UAS-GPI-Dfz2 lines were characterized and ranked according to the strength of the induced phenotypes. A strong UAS-Dfz2 insert on the third chromosome was used for all subsequent experiments except Figures 3A, 3B, 3J, and 3K, where an intermediate strength line was used. Three strong UAS-GPI-Dfz2 lines were used and produced identical results.

The UAS-arm^{act} line SC10 and the UAS-$dTCF^{DN}$ line ΔN4 expressing a deleted form of arm (Pai et al. 1997) or dTCF (van de Wetering et al., 1997) were obtained from M. Peifer.

wg^{IL} cn bw sp and *wg^{IL} br pr* mutant chromosomes, which are viable as transheterozygotes when reared at 16°C, were used to inactivate Wg signaling conditionally at 29°C. A SM5a-TM6B compound chromosome (from J. P. Couso and A. Martinez Arias) containing the dominant larval marker Tubby was used to identify the *wg^{IL}* homozygous larvae.

Ectopic Expression

The Gal4 drivers used were 69B- and 1J3-Gal4 (Brand and Perrimon, 1993), and 71B-, Ptc-, and En-Gal4 (Johnson et al., 1995). To avoid defects before the third larval instar, crosses with Ptc or 1J3-Gal4 to UAS-GPI-Dfz2 and En-Gal4 to UAS-$dTCF^{DN}$ were reared at 18°C and then shifted to 29°C for 24 hr prior to fixation. In all experiments, Gal4 drivers were crossed to UAS-lacZ (Brand and Perrimon, 1993) for negative controls and sometimes to mark the cells expressing Gal4. Unless otherwise noted, at least 15 discs of each condition were examined. Random clones placing Gal4 under the control of an Actin 5c promoter were generated by the strategy of Pignoni and Zipursky (1997) to express UAS-Dfz2 and UAS-GPI-Dfz2.

Loss-of-Function Clonal Analysis

To examine the loss of *wg* activity in a background misexpressing GPI-Dfz2, the following crosses were made: *yw* P[*hs-flp*]; P[*arm-lacZ*] *FRT40A*; P[*UAS-GPI-Dfz2*]/+ females crossed to *wg^{CX4} FRT40A; 1J3-Gal4*/SM5a-TM6B or *wg^{CX4} FRT40A, Ptc-Gal4*; +/SM5a-TM6B males. Half the progeny misexpress GPI-Dfz2, and all of the non-Tubby larvae have the potential to make *wg* clones, induced by Flp recombinase–induced crossover at FRT sites (Xu and Rubin, 1993) during first larval instar and marked by the absence of arm-lacZ staining (Pan and Rubin, 1995). The *wg^{CX4}* allele is a small deficiency that is RNA and protein null (van den Heuvel et al., 1993).

zw3 clones were made by crossing *$zw3^{M11}$ FRT18A*/FM7 females to P[*arm-lacZ*] *FRT18A*/Y; P[*hs-flp*] males and examining female progeny. *$zw3^{M11}$* is a strong allele (Siegfried et al., 1992), and the P[*arm-lacZ*] chromosome is described in Vincent and Lawrence (1994). *dsh^{75}* clones were made as described in Rulifson et al. (1996). Clones were induced during early second larval instar.

In Situ Hybridization and Whole-Mount Immunostaining

Dfz2 RNA and Wg antibody/*Dfz2* RNA double stainings were done as previously described (Cadigan and Nusse, 1996), except that the in situ hybridization procedure was performed as outlined in Kozopas et al. (1998). For fluorescent staining of *wg* transcripts, mouse anti-digoxygenin 1° antibody (Boeringher Mannheim) followed by anti-mouse Cy3 conjugated 2° antibody (Jackson Research) were used to detect digoxygenin-labeled *Dfz2* antisense probe. A detailed protocol is available upon request.

Whole-mount immunostaining was done as described previously (Cadigan and Nusse, 1996). Affinity-purified rabbit anti-wg antisera (1:50) was provided by C. Harryman Samos, purified rabbit anti-*Dll* antisera (1:150) was from G. Panganiban, mouse monoclonal anti-ac hybridoma supernatant (1:4) was from K. Vorwerk and S. Carroll, mouse monoclonal anti-cut hybridoma supernatant (1:20) was from K. Blochinger, and mouse anti-Delta ascites fluid (1:10,000) was provided by A. Parks and M. Muskavitch. Mouse anti-myc epitope monoclonal was from the University of Wisconsin Hybridoma Facility, rabbit anti-β-galactosidase antisera was from Cappel, and mouse monoclonal anti-β-galactosidase was from Sigma. FITC, Cy3, and Cy5 conjugated 2° antibodies were from Jackson Immunochemicals. All fluorescent pictures were obtained with a Bio-rad MRC-1000 confocal laser coupled to a Zeiss Axioscope and processed as Adobe Photoshop files.

Western Blot Analysis of Imaginal Discs

Discs were dissected from third instar larvae in cold PBS and transferred to eppendorf tubes, where they were homogenized in SDS loading buffer with tight fitting pestles, heated to 100°C for 4 min, and then stored at −20°C until use. Immunoblotting was done as described by Willert et al. (1997) using mouse monoclonal anti-Wg supernatant (1:100) provided by S. Cohen or rat monoclonal anti-α-catenin (1:1000; [Oda et al., 1993]) as a loading control. The secondary antibodies were conjugated to horseradish peroxidase and detected with SuperSignal ULTRA Chemiluminescent Substrate (Pierce) using the manufacturer's protocol.

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